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Interactions of Cardiac Glycosides with Na+K+ -ATPase Prepared from Several Guinea Pig and Rat Tissues

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INTERACTIONS OF CARDIAC GLYCOSIDES WITH Na^+K^+-ATPASE

PREPARED FROM SEVERAL GUINEA PIG AND RAT TISSUES

by

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A dissertation submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Graduate Studies.

Department of Biochemistry

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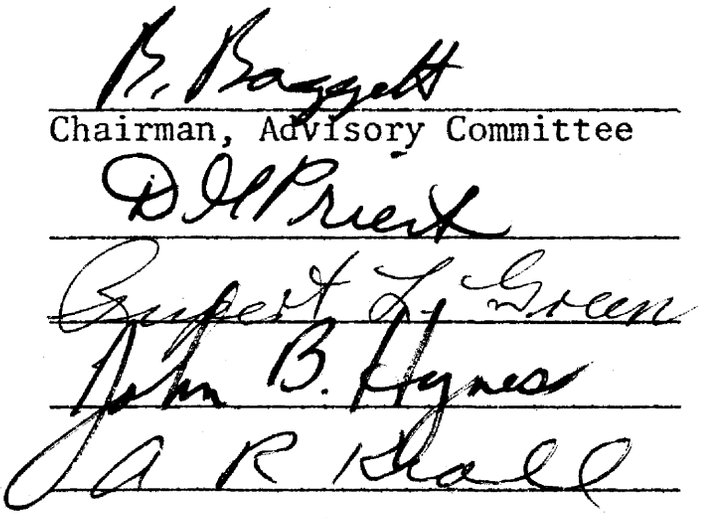
Approved by:



Chairman, Advisory Committee

SYLVIA AVELINE BRADDON. Interactions of Cardiac Glycosides with Na^+K^+-ATPase Prepared from Several Guinea Pig and Rat Tissues. (Under the direction of BILLY BAGGETT).

Interactions between five different cardiac glycosides, digitoxin, cymarol, convallatoxol, digoxin and ouabain, and Na^+K^+-ATPase from guinea pig heart, kidney, brain and rat heart and brain have been studied. Concentrations of glycosides required for 50% inhibition of the enzyme (I_{50}) have been determined at various concentrations of K^+. The binding of the glycosides to the enzyme preparations was studied using tritiated glycosides. ATP, Mg^{2+}, and Na^+ were required for specific binding. Through the use of Scatchard plots, a high affinity binding site was demonstrated for each guinea pig enzyme preparation, and the association constants (K_a) were measured for each of the glycosides.

Although rat brain was demonstrated to have Na^+K^+-ATPase with a high affinity binding site for glycosides, this was not found to be true of rat heart Na^+K^+-ATPase.

The initial concentration of each glycoside required to half saturate the high affinity site (B_{50}) was calculated. The effects of varying the concentration of K^+ on K_a and B_{50} was ascertained. In the presence of a low concentration of K^+ (0.625 mM), binding and inhibition were studied under identical conditions. The B_{50} and I_{50} values were similar to each other for each guinea pig enzyme and each glycoside. This established that with the guinea pig enzymes the binding observed was to the inhibitory site on the enzyme. Digitoxin,

cymarol and convallatoxol were bound more tightly and were more potent inhibitors than digoxin and ouabain with all enzyme preparations. The enzymes from the three guinea pig organs did not differ appreciably in their interactions with the glycosides.

Inorganic phosphate plus Mg^{2+} could replace ATP, Mg^{2+} plus Na^+ in supporting the binding of the glycosides, but in this case Na^+ as well as K^+ inhibited the binding. These data support the concept that Na^+-K^+-ATPase is the receptor for cardiac glycosides in the guinea pig and that the binding occurs reversibly to the inhibitory site on the enzyme when it is phosphorylated. The data also support the concept that the binding process and the hydrolytic reaction, promoted by K^+, compete for the phosphorylated enzyme in guinea pig Na^+K^+-ATPase.

The B_{50} and I_{50} values for rat brain ATPase were different, with 50% inhibition requiring 100 fold more glycoside than the amount required to obtain 50% saturation of the high affinity site. The B_{50} concentrations were consistent with guinea pig brain B_{50} concentrations not only in terms of relative order but also in magnitude. Since 50% saturation of this binding site was at a much lower cardiac glycoside concentration than the I_{50} concentration, it appears that this binding site is not related directly to the inhibitory site.

Rat heart Na^+K^+-ATPase was inhibited by glycosides, but the I_{50} concentrations required were 30-1000 fold greater than those required with guinea pig heart ATPase. This observation, coupled with the fact that no high affinity binding to this enzyme could be demonstrated, indicates that the rat heart contains a Na^+K^+-ATPase which is extremely glycoside insensitive (lacks a high affinity inhibitory site). This may explain the relative lack of pharmacological action of glycosides

on rat heart as compared to guinea pig heart.

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B_{50} - concentration of a compound producing 50% saturation of a binding site

CG - cardiac glycoside

DOC - sodium deoxycholate

I_{50} - concentration of a compound producing 50% inhibition

K_a - equilibrium constant for association

K_d - equilibrium constant for dissociation

k_a - rate constant of association

k_d - rate constant of dissociation

n - moles ligand bound per mole protein

NEM - N-ethyl maleimide

RBC - red blood cell

SAR - structure-activity relationships

INTRODUCTION

A. Nature of Na^+K^+-ATPase.

The enzyme Na^+K^+-ATPase (EC: 3.6.1.3), was first recognized by Skou in 1957 (1). He suggested that this enzyme was responsible for transmembrane transport of sodium and potassium coupled to the hydrolysis of ATP. It was subsequently shown that hydrolysis of ATP as catalyzed by this enzyme is inhibited by cardiotonic steroids (2,3), and that these steroids also inhibit Na^+ and K^+ transport (4). These findings strengthened the hypothesis that Na^+K^+-ATPase is part of the Na^+, K^+-transport mechanism. Repke and Portius (5,6) investigated the possibility that Na^+K^+-ATPase of myocardial cells might be the "receptor" involved in the action of cardiac glycosides on the heart. The interrelationships among cardiac glycosides, Na^+K^+-ATPase and Na^+, K^+-transport have interested many biochemists, physiologists and pharmacologists, an interest that has led to a very large volume of literature on the subject.

The following discussion will touch on a few of the high points of this voluminous literature, especially as is deemed important to the research described in this dissertation. Numerous reviews of the relationship of Na^+K^+-ATPase to the "Na pump" as well as detailed reviews and chapters on specific aspects of the enzyme system have been compiled (7-17). It is therefore not the objective of this introduction to provide a complete review work on the subject.

The "Na pump" is of prime importance to the functioning of

specific cells (nerve, muscle), to the maintenance of osmotic pressure of all cells and to the ability of various tissues and organs (kidney, intestinal mucosa, secretory glands) to carry out their particular functions. The pump has many features that parallel those of Na^+K^+-ATPase. These have been reviewed concisely by Hokin in Metabolic Transport (7). The common features are: both require the simultaneous presence of Na^+ and K^+ in approximately equivalent half-maximal activation concentrations; both are inhibited by cardiac glycosides with approximately the same concentrations for half-maximal inhibition; the quantitative distributions of both are in good agreement; both seem to display sidedness in the membrane with Na^+ activating on the inside and K^+ activating on the outside; both the pump (18) and Na^+K^+-ATPase have been driven backwards to produce ATP-γ-^{32}P from $^{32}P_i$ and ADP (19). With these common features in mind it is difficult to overestimate the importance of Na^+K^+-ATPase to cell and tissue function.

Na^+K^+-ATPase appears to be an enzyme system which is an integral part of the plasma membrane. This has been most clearly demonstrated in the red blood cell (7). It can be characterized as an enzyme which has at least two types of subunits. One subunit appears to be a glycopolypeptide with a molecular weight of 55,000 and the other, a polypeptide associated with phospholipid with an estimated molecular weight of 84-105,000 (7,20). The subunits, even in the enzyme's most purified form, tend to cluster in vesicles similar to those seen in crude preparations of plasma membranes (20,21). The catalytic subunit of Na^+K^+-ATPase has been identified by labeling the aspartyl residue at the active site with ATP-γ-^{32}P in the presence of Na^+ and

Mg^{2+} (7,22). The molecular weight of the catalytic subunit is estimated to be 100,000, and it is probably the phospholipid containing subunit (7). As might be expected, the molecular weight of the enzyme system reported depends upon the methods used to purify the enzyme, the source of the enzyme and the method of determination.

Since Na^+K^+-ATPase is an integral part of the plasma membrane, there is some question as to whether the enzyme will ever be purified into discrete protein components that will show activity. There is also a distinct possibility that the enzyme system cannot be truly solubilized except in association with detergents. Determination of the physical properties of the enzyme often depend upon its solubilization, but the bound detergents will significantly alter any results obtained on such a preparation. Even without the involvement of detergents, associated carbohydrates and phospholipids will distinctly alter the properties of the enzyme in solution (23). To complicate matters further it has not been established that there is a constant carbohydrate composition of the Na^+K^+-ATPase. The estimates of the percentage of the total ATPase protein represented by the protein of the glycopolypeptide varies from 19 to 28% (21). It appears, on the basis of phospholipase treatment and phospholipid replacement, that a constant ratio of phospholipid is not necessary for enzymatic activity (24). Whether a parallel can be drawn, from these observations to the phospholipid and carbohydrate requirements in the membrane for Na^+K^+-ATPase activity, is not clear. Thus it is apparent that, although work on the purified enzyme will answer many important questions, methods devised to elicit information from the "membrane bound" enzyme will also prove invaluable. To this end it

is interesting to note that kinetic properties of the impure membrane bound enzyme are very similar to those for a highly purified Na^+K^+-ATPase (25).

In the isolation of Na^+K^+-ATPase there is always an associated activity of Mg^{2+}-dependent ATPase which is usually separated from Na^+-K^+-ATPase by differential centrifugation and deoxycholate treatment (7). The purification of Na^+K^+-ATPase has taken many routes (protease treatment, phospholipase treatment, physical disruption, detergent solubilization, salt fractionation) but most follow a general scheme. A typical scheme for the purification of Na^+K^+-ATPase is found in Table 1. Chaotropic agents such as NaI (7) are thought to extract some protein from microsome fractions; for example, the almost complete removal of Mg^{2+}-ATPase has been claimed by this method (7, 27). Deoxycholate, an anionic detergent, is often used in sub-solubilizing concentrations to remove inert protein, reduce the level of Mg^{2+}-ATPase and activate Na^+K^+-ATPase (7). Lubrol and SDS are also claimed to activate the enzyme; it appears that this is accomplished at critical micelle concentrations of the detergents. Other steps used in various procedures include ammonium sulfate fractionation, agarose chromatography, sephadex chromatography, CMC (carboxymethyl cellulose) chromatography, DEAE-cellulose chromatography, sucrose density gradient centrifugation, and glycerol gradient centrifugation.

Standard methods for the determination of the molecular weight have not usually been employed because of the lipid and carbohydrate contributions to the subunits. SDS polyacrylamide gel electrophoresis has been used most often to estimate the subunit molecular weights, although the same problem exists. Because of the considerable

TABLE 1

Purification of Na^+K^+-ATPase from beef brain microsomes[a]

Step	S.A. (μmoles/mg Pro/hr)	Total Activity (μmoles P_i/hr)	Total Protein (mg)
Microsomes	14.8	15,100	1020
NaI-treated microsomes	38.7	13,600	352
Lubrol-treated NaI-microsomes	28.3	8,210	290
Lubrol Extract	43.8	2,280	52
Pellet [b]	117.	1,560	13.4
CMC column eluate	153	1,150	7.5

[a] from Kahlenburg et al., 1969 (26).

[b] transparent, colorless, gelatinous pellet was obtained after centrifugation at 115,000 x g for 18 hr. It readily redissolved in buffer.

inhomogeneity of most preparations prior to the last year or two, the use of radiation inactivation and gel filtration were often used to determine the molecular weight of the enzyme (7). The former method has yielded a molecular weight of 250,000 for Na^+K^+-ATPase derived from human red blood cell, guinea pig kidney cortex and crayfish nerve cord plasma membranes (7). Lubrol and DOC treated microsomes quite regularly give a molecular weight between 500,000 and 700,000 as measured by gel filtration chromatography (7). Using the constant protein content observed throughout their preparation, Uesugi et al. (27) made corrections on the molecular weight of detergent isolated enzymes which accounted for the bound detergent, carbohydrate, cholesterol and non-phospholipid. When such corrections were made on a molecular weight determined by gel filtration of a detergent isolated enzyme, the calculated protein molecular weight was approximately 260,000 (based on a protein content of 55%).

As mentioned earlier the purified enzyme system apparently contains carbohydrate and phospholipid. Carbohydrate has been identified by use of the periodic acid-Schiff reagent (20), but not quantified. According to Simpkins and Hokin (21) the purified enzyme from the rectal salt gland of Squalus acanthias contains protein and phospholipid in a ratio of 2:1, but data are not given. Other reports of lipid content are variable, dependent upon the mode of isolation. A major question arises: Are carbohydrates and phospholipids necessary and functional components of the ATPase system? The role of carbohydrate has not been thoroughly investigated and is not as yet clear. The function of phospholipids on the other hand has been studied by many research groups (7,9,12,13). Most of the work

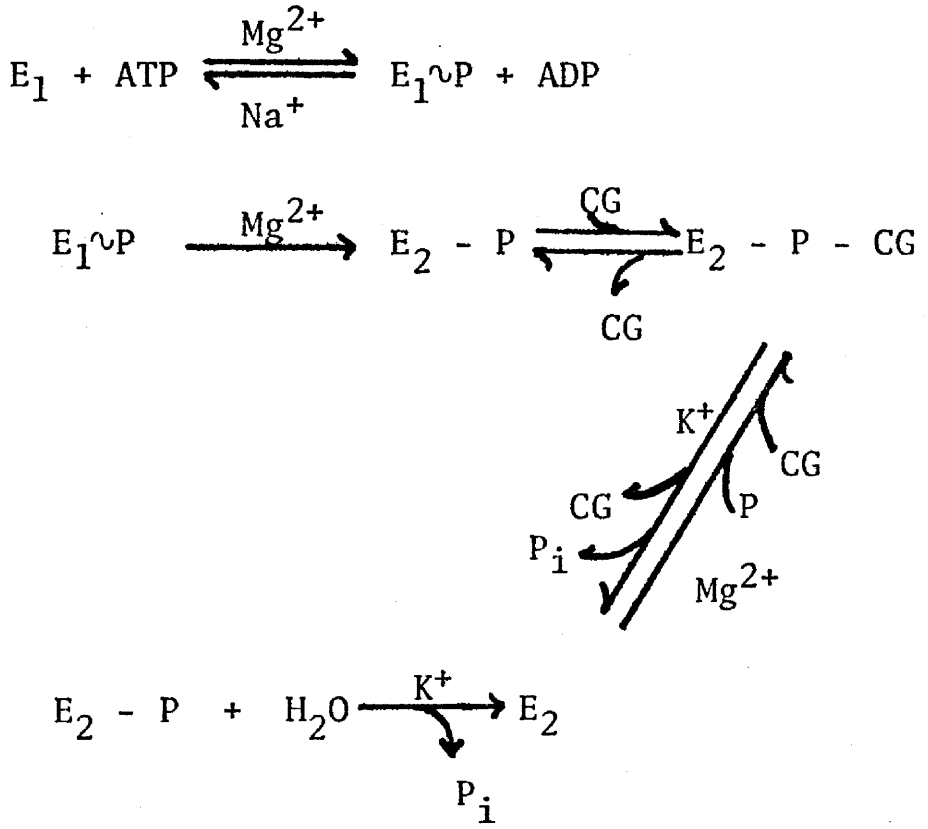
to date has consisted of phospholipid removal with solvents (7), detergents (28,29) or phospholipases (14,15). The resultant inactive Na^+K^+-ATPase then is treated with various phospholipid preparations and individual phospholipids (phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl choline) in an attempt to restore activity. Variations in time of extraction, time of reactivation treatment, purity of the particular phospholipid used for reactivation treatment and purity of the particular phospholipid used for reactivation and inhibition by released fatty acids (30,31) have led to conflicting results. Several claims have been made that phosphatidyl serine is the activating lipid (7,32,33), but a systematic investigation (35) of Na^+K^+-ATPase phospholipid requirements revealed that a minimum structure of a phosphate plus two fatty acyl residues is required. It appears that phosphatidyl ethanolamine, phosphatidic acid and phosphatidyl inositol will reactivate as well as phosphatidyl serine (7). It has been suggested that phospholipid reactivation may be the result of replacement of detergent and lyso-derivatives which can inactivate the enzyme (31). Recent work employing spin-labeling techniques and x-ray diffraction studies indicate that the phospholipid in highly purified Na^+K^+-ATPase possesses bilayer character. Inactivation experiments with phospholipase A using N-ethylmaleimide spin-labeled enzyme indicated that a conformational change in protein occurs as the result of disruption of the lipid bilayer. It can now be concluded that the role of phospholipids is not well defined, but that the evidence points towards a role in the maintenance of the protein in an appropriate conformation (7,30-35).

Na^+K^+-ATPase hydrolyzes ATP to yield ADP plus P_i. This process

requires Na^+, Mg^{2+}, and K^+. Several enzyme sites have been characterized for Na^+K^+-ATPase. When the enzyme system is considered in the RBC membrane, it displays sidedness; therefore, the sites can be oriented in relation to the intra- and extra-cellular fluids. It appears that the ATP site is on the inside of the cell membrane along with a site having an affinity for Na^+ which is 4-6 times the affinity for K^+ (13,35). There is a monovalent cation site on the outside of the membrane which has an affinity for K^+ that is 60-100 fold that for Na^+, but that will accept other ions (i.e. $K^+ > Rb^+ > NH_4^+ > Cs^+ > Li^+$) (13,35). The requirement for Na^+ and Mg^{2+} are absolute, if overall enzymatic activity is to be observed. The interrelationships of Na^+ and K^+ especially are very complicated as numerous kinetic studies have pointed out (10). In a system with at least four required components (excluding the enzyme itself) the smallest changes in concentration of any or all can have considerable effect on the enzymatic activity. Mg^{2+} may have more than one role, in that the enzyme may have sites requiring Mg^{2+}, besides the site which binds Mg^{2+}-ATP. It appears now, after considerable study into the reaction mechanism, that the enzyme acts in a multistep manner with several steps defining the "phosphorylation phase" of the enzymatic activity and at least one step involved in the "dephosphorylation phase" (7,36-41). There is not a complete solution to the exact reaction mechanism for Na^+K^+-ATPase, but from many approaches to the problem the following simplified picture arises (see Figure 1). There have been many imaginative approaches taken to elucidate the experimental evidence for the reaction sequence of the hydrolysis of ATP by Na^+K^+-ATPase; but, of the numerous studies done, several stand out. In support of a phosphory-

Figure 1.

Proposed Enzyme Form Involved in the Interactions of Na^+K^+-ATPase with ATP and Cardiac Glycosides



lated intermediate a Mg^{2+}-dependent ATP-ADP exchange was described (7). Furthermore many researchers (7) reported a very rapid Na^+-dependent incorporation of ^{32}P from ATP-γ-^{32}P in the presence of Mg^{2+}, the ^{32}P remaining bound to the protein even after TCA precipitation. K^+ included in the above experiments greatly diminished the level of ^{32}P in the TCA precipitated protein (7). In conflict with the ADP-ATP exchange data of Skou were several pieces of evidence that the bulk of the exchange could apparently be separated from Na^+K^+-ATPase hydrolytic activity (7).

Pursuant to these studies were those that provided evidence that there is more than one phosphorylated form of Na^+K^+-ATPase. Several groups of researchers (37-40) demonstrated at low Mg^{2+} concentrations (as compared to those required for hydrolysis) an exchange activity which has an absolute specificity for Na^+ and ATP, a non-dependence on K^+, and is inhibited by cardiac glycosides. It was postulated that one phosphorylated form of the enzyme, $E_1 \sim P$, was converted to another conformation, E_2-P, via Mg^{2+}, an essentially irreversible reaction, and that the E_2-P form could not react with ADP. Therefore, under conditions leading to hydrolysis, namely at high magnesium levels, the form $E_1 \sim P$ would be but a fleeting intermediate.

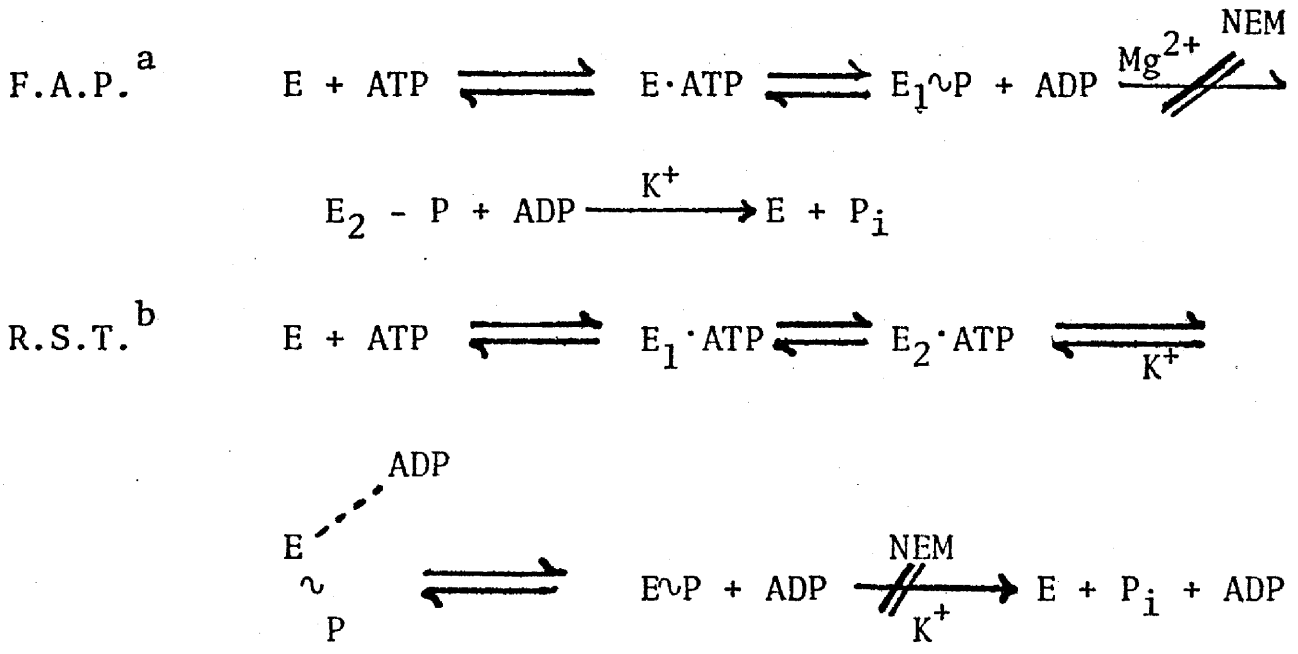
Another approach to the problem of defining discrete partial reactions in the overall enzyme mechanism has been the use of inhibitors. Most inhibitors function after the phosphorylation partial reaction, most likely at the step where E_1 is converted to E_2. The exception to this is that at high concentrations of glycosides and in the absence of Na^+ an inhibition of the ATP-ADP exchange reaction is observed (7).

Support for the existence of the two phosphorylated enzyme forms $E_1 \sim P$ and E_2-P arises from studies with various inhibitors (N-ethylmaleimide, oligomycin, arsenite and 2,3-dimercaptoethanol) which block E-P hydrolysis (10,39,41). In the blocked enzyme there appear to be two phosphorylated forms of the enzyme with differing sensitivities to several ligands. A comparison of the phosphorylated forms of the enzyme in the native and NEM-treated states leads to the following observations: the native phosphorylated enzyme is hydrolyzed in response to K^+ but not ADP, whereas the NEM-treated phosphorylated enzyme is dephosphorylated in response to ADP but not K^+ (7,37-39). Peptide digests of these phosphorylated forms of the enzyme display no differences. It has been concluded from this type of study that the change in phosphorylated forms may simply represent a conformational change (7,36).

An alternate sequence of partial reactions has been proposed for the mechanism of Na^+K^+-ATPase (42). In a recent paper Fukushima and Tonomura (43) have elaborated on the earlier proposed mechansim and have provided additional evidence to support the modified K.S.T. (Kanazawa, Saito, Tonomura) mechanism. The major differences between the K.S.T. mechanism and that proposed by Fahn, Albers, Post and coworkers (37,38) (F.A.P. mechanism) are (see Figure 2): 1) the existence of a high energy intermediate (in the presence of high Mg^{2+} concentrations) which will lead to the formation of ATP upon the addition of K^+ in addition to the expected breakdown of high energy intermediate to yield P_i; 2) the existence, in the presence of low Mg^{2+} concentrations and in an NEM-treated enzyme, of two high energy phosphorylated intermediates, with and without bound ADP. Using a

Figure 2.

Postulated Reaction Mechanisms
for Na^+K^+-ATPase



[a] Scheme of Fahn, Albers and Post (37,38).

[b] Scheme of Kanazawa, Saito and Tonomura (42).

special apparatus that allowed for split second reaction times, good evidence was provided to demonstrate that one intermediate was sensitive and the other insensitive to the addition of ADP.

In support of the F.A.P. model, Arrhenius profiles have been used to support possible conformational changes in Na^+K^+-ATPase. Two distinct discontinuities appeared at 6° and 20° in the Arrhenius plot of rat brain Na^+K^+-ATPase (44).

Another approach to the detection of conformational changes has been the use of fluorescence probes. 1-Anilino-8-napthalenesulfonic acid has been used for such studies, since it responds to increased hydrophobicity in its environment by a decrease in the wavelength of its maximum emission (7). The results are cloudy, since it now appears that many of the observed effects are nonspecific (7); thermal inactivation of the enzyme did not affect the fluorescence changes observed with the ligands ATP, Na^+, and K^+. Hellebrigenin-1,4-sulfonaphthyl-hydrazone and the corresponding strophanthidin derivative were used in attempts to find a site specific probe (45). Both were biologically active as cardiotonic steroids and displayed some interesting ligand effects. Na^+ enhanced the fluorescence while K^+ and other monovalent cations which substitute for K^+ suppressed it. The order of effectiveness of the suppressing ions ($K^+ > Rb^+ > Li^+ > Cs^+ > Tl^+ > NH_4^+$) paralleled the order of affinities for the K^+ site. Other effects were observed with various combinations of ATP, Mg^{2+}, P_i and monovalent cations which all lead to the notion that there are conformational changes induced by these ligands for the enzyme. The effects could be abolished by low pH denaturation or interaction with the inhibitor DFP, and, most importantly, were antagonized by

competition with hellebrigenin for the active site.

The phosphorylated residue appears by recent evidence to be the beta carboxyl of an aspartic acid residue (46). It had previously been reported by Trevor, Rodnight, and Schwartz in 1965 (47) that a serine residue was the amino acid phosphorylated during ATP hydrolysis, and more recently by Kahlenburg et al. (48) that it was the gamma carboxyl of a glutamate residue. A recent paper employing the use of ^{3}H-borohydride reduction of the acyl phosphate linkage and identification of the resulting ^{3}H-ω-hydroxyamino acid formed confirms (Post et al.) the observations that the β-carboxyl group of an aspartic acid residue is phosphorylated (49).

B. Cardiac Glycosides.

The cardiotonic steroids (also called cardiac glycosides or 'digitalis') are powerful inhibitors of $Na^{+}K^{+}$-ATPase (50). They have been isolated from a wide variety of plants (leaves, flowers, seeds, stems, roots, and bark all serve as the source) and from the secretions of toads (51). The cardiotonic steroids have served as medicines and as arrow poisons for centuries. The Scottish physician William Withering introduced the use of digitalis, an extract from the dried seeds and leaves of purple foxglove, in the late 1700's for the treatment of "dropsy" (17,51). His careful study of over 200 patients set the stage for continued use of digitalis drugs in the treatment of cardiac patients. Although Withering recognized the powerful action of the drugs on the contraction of the heart, enlightenment as to the drugs' actual function has only occurred in the mid-1900's. Their specific pharmacologic activity is to increase the force of contraction of the heart muscle, diminish the heart rate

and improve cardiac efficiency. Even today the mechanism by which the cardiotonic steroids increase the force of contraction of the heart is hotly debated. Recently the use of radio-labelled cardiac glycosides has made feasible the study of the distribution of the drug in various tissues of the body. Short term exposure to the drug doesn't show a favored uptake by the heart, but in six hours the heart (of a sensitive species) has retained most or all of the glycoside taken up (17) while blood levels have dropped drastically. It is accepted by most workers in the field that the specific receptor for glycosides in most tissues studied is Na^+K^+-ATPase; the controversy arises over heart tissue for which there are factions divided over the mode of action of cardiac glycosides. One group postulates that Na^+K^+-ATPase is the primary receptor for the cardiotonic steroids (52) and that their mode of action in increasing force of heart muscle contraction is through ionic changes caused by inhibiting the "Na pump". This inhibition leads to increased intracellular Na^+ which causes changes in the distribution of Ca^{2+} within the cell. Another view is that the Ca^{2+} redistribution is a more direct effect of a conformational change of Na^+K^+-ATPase in the membrane upon the binding of cardiac glycosides independent of the Na^+ effects (53-55). Finally, it is postulated that cardiac glycosides bind to other receptors in addition to Na^+K^+-ATPase and this binding influences the contractile process (17).

The basis for the research described here stems from the above controversy. The objective was to compare a drug-sensitive species, guinea pig, with an insensitive species, rat, in terms of the interactions of Na^+K^+-ATPase with cardiotonic steroids. Further, the goal

was to characterize Na^+K^+-ATPase from several tissues of the sensitive and insensitive species in terms of their binding and inhibition parameters for various cardiac glycosides.

In addition to the study of the mode of action of cardiac glycosides in terms of their positive inotropic effects, cardiac glycoside/Na^+K^+-ATPase interactions have been used in experiments on the partial reactions of ATP hydrolysis (see earlier discussion). These studies are of particular interest to the biochemist-pharmacologist as a model for drug-receptor interactions.

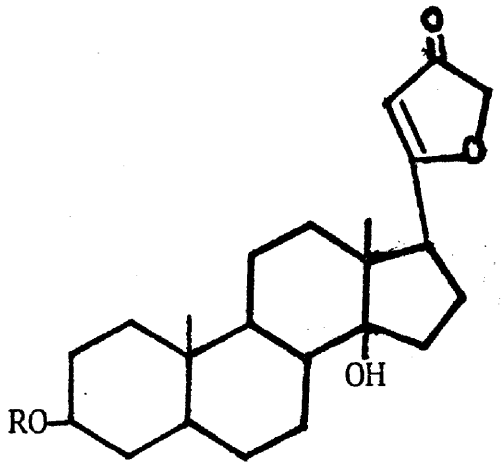
Many interesting observations have been made on structure-activity relationships (SAR) between cardiac glycosides and Na^+K^+-ATPase. The cardiac glycosides fall into two groups, the cardenolides and the bufadienolides (see Table 2), with the latter being generally more potent inhibitors. Structural features required for binding and inhibitory activity are: 1) an unsaturated lactone ring in the beta configuration at C-17 of the steroid nucleus; 2) a cis ring juncture between the A and B rings of the steroid nucleus; 3) a beta-hydroxyl at C-14; and 4) a beta-hydroxyl or beta-glycosidic linkage at the C-3 position (7,55,60). The cardiac glycosides presented in this dissertation are listed in Table 2.

Until very recently the best SAR studies made on cardiotonic steroids were done by toxicity assay (8,58,64) or inhibition-toxicity comparison studies. In 1966, Repke and Portius (64) reported SAR studies for a large number of cardiotonic compounds based on their inhibition of Na^+K^+-ATPase. They pointed out the requirement of a carbonyl function conjugated with a double bond for inhibitory activity. The erythrophleum alkaloid, cassaine, neither possesses a steroid

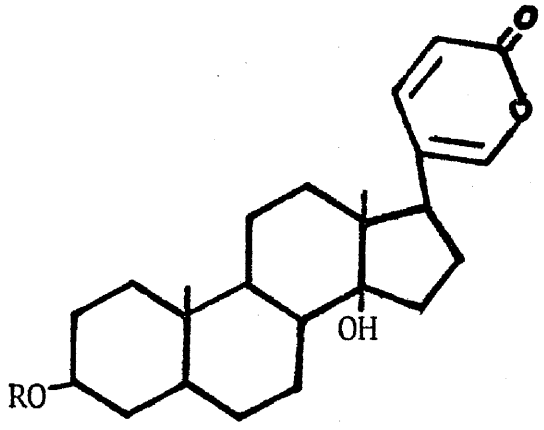
TABLE 2

Cardiac glycosides of two classes

cardenolide (c)



bufadienolides (b)



Steroid	R	C_{19}	Substitution	Class
Convallatoxol	L-rhamnosyl	CH_2OH	5β-OH	c
Cymarol	D-cymarosyl	CH_2OH	5β-OH	c
Digitoxin	(3)D-digitoxosyl	CH_3	-	c
Digoxin	(3)D-digitoxosyl	CH_3	12β-OH	c
Hellebrin	D-rhamnosyl + D-glucosyl	CHO	5β-OH	b
Ouabain	D-rhamnosyl	CH_2OH	1β,5β,11α-OH	c
Strophanthidin	H	CHO	5β-OH	c

nucleus nor an unsaturated lactone (as do cardiac glycosides) yet it inhibits Na^+K^+-ATPase (I_{50} = 0.5 μM); however, it does have a carbonyl conjugated with a double bond in its side chain. They also point out that the oxygen function at C-3 is important to inhibitory activity, but that the sugar component doesn't appear to be crucial.

When approaches were developed to study the mechanism of the cardiac glycoside/enzyme interaction, hypotheses were advanced to explain the SAR. In 1970 Wilson et al. (56) postulated a binding site model for cardiac glycoside/Na^+K^+-ATPase interactions which was comprised of three components. The A component reacted optimally with the sugar portion of an aglycone monosaccharide; the B component interacted with the 14-hydroxyl group on the steroid nucleus; and the C component interacted with the lactone ring with preference for the bufadienolide type. Applying Wilson's model, Yoda in 1973 studied the SAR involved in the dissociation rates of various cardiac glycoside/enzyme complexes (57). He found that the dissociation rate of the complex was dependent upon the sugar portion of the cardiac glycoside and temperature, but was independent of the aglycone. He suggested from his data that the rate determining step of the dissociation might be a conformational change that occurred accompanying the dissociation of the sugar portion but prior to the dissociation of the steroid. In a subsequent paper Yoda and coworkers (65) described SAR studies employing association rates. It was concluded from these experiments that the steroid portion of the cardiac glycoside binds first and determines the k_a. A conformational change following steroid binding and preceding sugar binding was proposed. Differences between monoglycosides and oligosaccharides of the same steroid are explained

by the effect of differences in the bulk effect of the sugar moiety.

C. Na^+K^+-ATPase/Cardiac Glycoside Interactions--Inhibition.

Cardiac glycosides inhibit the hydrolysis of ATP by Na^+K^+-ATPase. The rate of such hydrolysis and consequently the degree of inhibition can be measured by determining the rate of release of P_i from ATP under suitable conditions. Ten millimolar glycoside will totally inhibit all known Na^+K^+-ATPases. Some preparations are fully inhibited by concentrations as low as 1×10^{-4} M. Concentrations of glycoside lower than the fully inhibitory ones bring about degrees of inhibition that are related to the concentration. Within the range of inhibitory concentrations the degree of inhibition is proportional to the logarithm of the glycoside concentration. Cardiac glycosides interact with Na^+K^+-ATPase at a site which is specific for cardiotonic steroids and is thought to be located on a hydrophobic protein, possibly the glycoprotein subunit (66). Most of the knowledge on this interaction has been elucidated, up until fairly recently, by studying the inhibitory effects of cardiac glycosides on Na^+K^+-ATPase. Inhibition is time, temperature, glycoside concentration, ligand concentration, tissue and species dependent. This complexity, in all probability, explains the diversity of the experimental observations--large variations in observed I_{50} values, claims of irreversibility versus reversibility of the enzyme glycoside interaction, the questioned necessity of a phosphorylated form of the enzyme to which the glycoside binds, whether K^+ and glycosides compete for the same binding site.

It has generally been accepted that in the assay of Na^+K^+-ATPase the Na^+/K^+ ratio should be between 5/1 (67) and 10/1 (1) with the Na^+ level at 100 mM, and that the Mg^{2+} concentration should be at least

twice that of ATP (7,68). It would appear, though, that the maximum inhibition by cardiotonic steroids occurs at Na^+/K^+ ratios much higher, i.e. 100/1 (69). If the concentration of potassium is reduced, the I_{50} value is also reduced (8,67,70). Sodium increases at constant K^+ concentrations also lead to an increase of the I_{50} value (69). Matsui and Schwartz (69) concluded from their kinetic studies of the effects of Na^+ and K^+ on the inhibition that it was impossible to separate the two effects and that the Na^+/K^+ ratio was the important determinant. The K^+ effect is not thought to be a competition between cardiac glycoside and K^+ for a particular site as was earlier postulated (67). It has more recently been postulated by Wolf and Peter (71) that the binding of K^+ induces a conformational change which decreases the binding of ouabain. This conclusion was arrived at on the basis of an observed partial competition between K^+ and ouabain and the assumption that the relative degree of ouabain inhibition is independent of the Na^+ concentration. The differences in I_{50} values for various enzyme preparations reported in the literature may be a result of not only differing affinities for various glycosides, but also the result of different affinities of the enzyme for K^+ and/or Na^+ (71).

Further evidence of the occurrence of a conformational change as the result of K^+ binding to the enzyme is provided in studies employing F^- as an inhibitor (72). The fluoride-inhibited enzyme can be reactivated at a much faster rate than K^+-complexed fluoride inactivated enzyme, which suggests that a change in conformation occurs when the inhibited enzyme is exposed to K^+.

An unusual phenomenon which has been observed is the stimulation

of Na^+K^+-ATPase by cardiac glycosides at low concentrations (usually less than 1 x 10^{-8}M) (8,11). This stimulation is not altered by varying the K^+ concentration from 0.01 - 25 mM (11). It has been suggested, as an explanation, that low ouabain concentration affects the conformation of the enzyme in a way dependent upon pre-reaction conditions (11).

An interesting observation was made by Godfraind and DePover (73) when I_{50} values for guinea pig heart ATPase (0.22 μM) were compared to the values for half saturation of the atria (0.21 μM), fifty percent inhibition of K^+ uptake (0.24 μM), and fifty percent inotropic effect (0.28 μM) caused by digitoxin. The authors suggest that these results are indicative of at least a superficial relationship between Na^+K^+-ATPase inhibition and the cardiotonic effects of the glycosides.

There is great variation in species sensitivity to cardiac glycoside inhibition (74). At divergent ends are the rat and toad (insensitive species) as compared to the guinea pig, dog, cat, frog, pigeon and human (sensitive species). The I_{50} values for the rat and toad cardiac Na^+K^+-ATPases are in the range of 0.7 - 1.0 x 10^{-4} M ouabain (74). The corresponding values for the sensitive species are as low as 5 x 10^{-8} M and as high as 7 x 10^{-7} (74). Repke demonstrated a 100 fold difference in the inhibitory concentrations for rat and guinea pig cardiac Na^+K^+-ATPases (70,74). Allen and Schwartz (75) offered as a possible explanation that the glycoside-enzyme complex formed by Na^+K^+-ATPase from a sensitive species was more stable than the complex formed by the enzyme from an insensitive species. Since this conclusion was drawn from binding studies which were carried out under high concentrations of glycoside there is some question as to

validity.

D. Binding Methods and Experimental Data Interpretation.

Binding of a ligand by a protein can be measured in many different ways. The choice of the method is dependent upon the chemical and physical characteristics of not only the binding protein but also of the ligand. Several generally used methods cited in Edsall and Wyman (76) are: equilibrium dialysis, ultrafiltration, spectroscopic measurements, electrophoretic mobility studies, and pH changes associated with binding. The list given is by no means all-inclusive. The list does leave out a distinctly useful method which is dependent upon an easily pelletable protein and a radioactively labelled ligand. The latter method allows quantification of the bound species by radioisotopic detection and is dependent upon separation of the bound ligand from the unbound ligand by centrifugation of the pelletable protein followed by physical separation of the two phases. Since the studies herein were carried out on a partially purified membranous protein, the latter method proved the most convenient to use for the study of the binding of cardiac glycosides-^{3}H by ATPase. Large numbers of samples could be handled simultaneously. This allowed the study of not only several glycosides under a multiplicity of conditions but also the comparison of ATPase from several tissue sources under various conditions.

For a binding process, the association constant (K_a), is obtained from the expression, $K_a = \frac{b}{(n-b)[u]}$, where b = bound ligand, n = number of binding sites and [u] = concentration of free ligand.

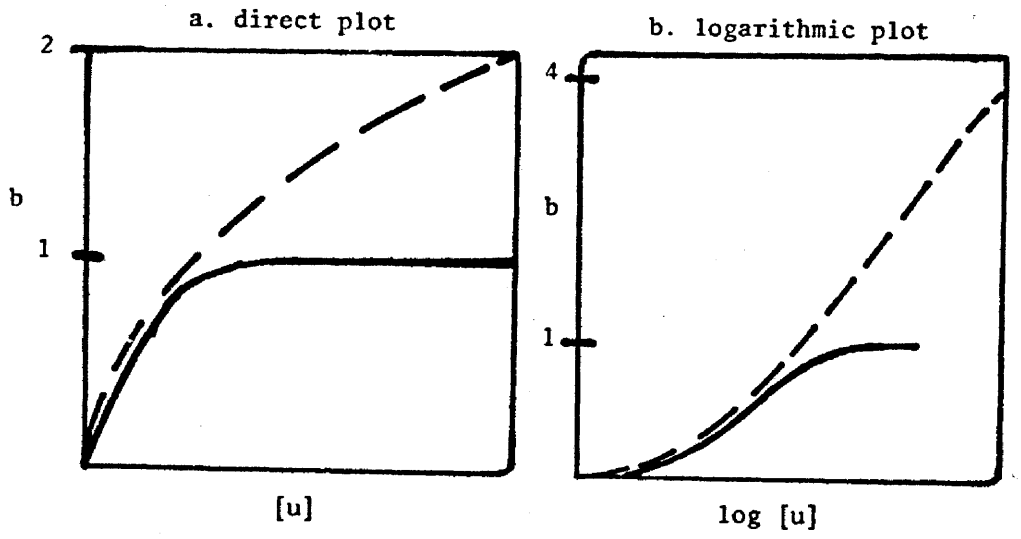
The analysis of experimental binding data obtained using one of the above methods can be accomplished by the use of one of the follow-

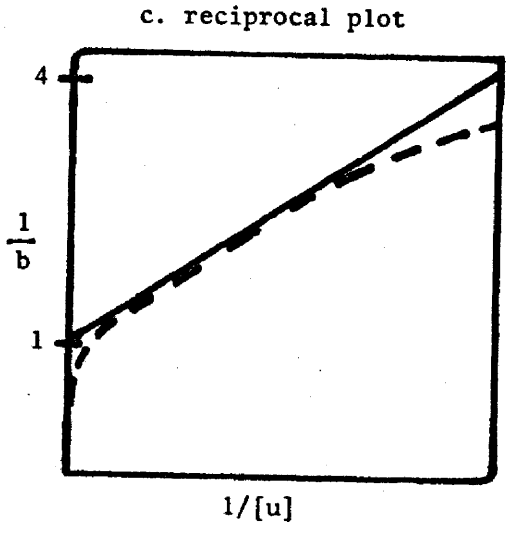
ing methods of plotting the data. Each of these approaches will allow the determination of at least two binding parameters, i.e. the affinity constant (K_a) and the number of moles ligand bound per mole of protein (n). The methods and their axis determinants are; direct plot, b vs [u]; logarithmic plot, b vs log [u]; reciprocal plot, 1/b vs 1/[u]; Scatchard plot b/[u] vs b.

The "direct plot" (76) (see Figure 3a) is the simplist way to analyze experimental binding data for a single set of binding sites (76). It is represented by the following formula: $b = \frac{nK_a[u]}{1 + K_a[u]}$, and appears as a rectanglular hyperbola for a single set of binding sites. The curve asymptotically approaches the horizontal where b=n is the limiting case. Thus K_a can be determined from the unbound concentration at b = n/2. If there is more than one set of binding sites the curve does not follow the "ideal" rectangular hyperberola, but instead approaches the horizontal much more gradually (see Figure 3a). This makes the determination of the line b = n difficult and therefore the values for n and K_a quite inaccurate. This is the actual case in most attempts to use this method, and therefore highly accurate values are usually not obtained.

The logarithmic plot (76) (see Figure 3b) is simply a titration curve where a wide range of unbound ligand ([u]) may be plotted. For a single set of equivalent sites the following equation expresses the logarithmic plot: $\log K_a + \log [u] = \log \frac{b}{n-b}$. The K_a is obtained from the value of log [u] when b = n/2 and is expressed in the equation as $-\log K_a$. n is not accurately obtained by this method; therefore, if the n value is not already known, the K_a cannot be accurately obtained either. The method may be used to determine whether one, or

Figure 3. A graphical display of three methods for plotting experimental binding data: direct plot, logarithmic plot and reciprocal plot (76).





one set equivalent binding sites

two sets equivalent binding sites

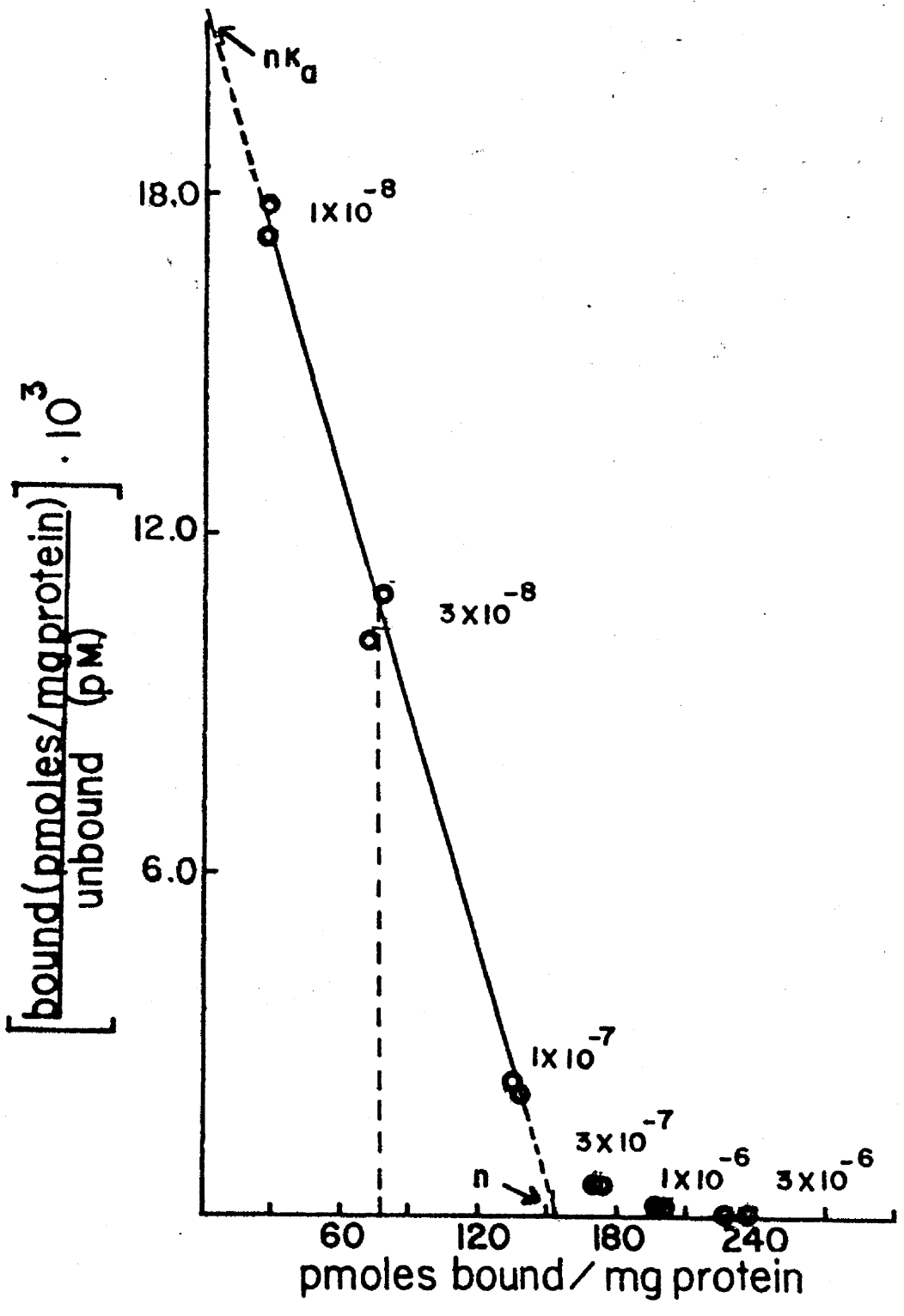
more than one, class of binding sites is represented by particular data, and an approximate n may be determined for such data from the shape of the curve.

The reciprocal plot (76) (see Figure 3c) is another method of plotting binding data which best deals with single sets of equivalent binding sites. From the equation for the reciprocal plot, $1/b = 1/n + 1/n\ K_a\ [u]$, it is seen that both n and K_a may be determined directly where 1/n is the intercept on the "y" axis and the slope is given by $1/K_a n$. If an attempt is made to use this plot on data which arises from two sets of equivalent sites, interpretation becomes difficult. It is only at high unbound concentrations that a marked deviation from linearity should be observed. This is an area of the plot where it is difficult to differentiate normal data scatter from the deviation from linearity that should be observed as a result of a second set of equivalent binding sites. Therefore, in practice, it is difficult to know whether the deviation from linearity is actually present.

The last method to be considered is the Scatchard plot (76) (see Figure 4), which can be represented by the following expression, $b/[u] = K_a\ (n-b)$. The Scatchard plot was chosen to represent the experimental binding data in this work since it yields the best estimates of the affinity constant, K_a, and the number of binding sites, n, where more than one class of binding sites exists. This mode of expressing the data places less emphasis, than the reciprocal plot, on values of b obtained at very low [u] values and more evenly weights different portions of the curve (76). Therefore, even though there may be some difficulty in extrapolating from a conspicuously non-linear curve, the Scatchard plot still allows good approximations of n and K_a

Figure 4. Example of Scatchard plot: Binding of convallatoxol-^{3}H to $Na^{+}K^{+}$-ATPase.

The number beside each set of data points refers to the total molar concentration of convallatoxol. The dashed line (---) is drawn from the value n/2 up to the curve; from this intercept the B_{50} concentration can be calculated using the Scatchard relationship. $\underline{n}$, $\underline{nk_a}$, and B_{50} are defined in the text.


nK_d
18.0
12.0
6.0
1X10^-8
3X10^-8
1X10^-7
3X10^-7
1X10^-6
3X10^-6
n
60
120
180
240
pmoles bound / mg protein
[bound(pmoles/mg protein) / unbound (pM)] · 10^3

to be made from non-linear curves.

In the present work n (Figure 4) is taken to be a relative value describing the number of binding sites per mg protein, and nK_a divided by n yields K_a, the affinity constant or "intrinsic association constant" (76) for the binding site. The association constant, K_a, then describes the affinity of the ATPase binding site for the ligand, CG. Therefore, comparisons between K_a values for different cardiac glycosides should yield the relative affinity of the enzyme, Na^+K^+-ATPase, for these glycosides.

If n is the amount of CG bound at full saturation of a specific set of binding sites, then n/2 describes the amount of CG bound at half saturation. In the Scatchard plot shown (Figure 4), it is apparent that the value of n/2 is achieved by a total CG concentration near 3×10^{-8} M. This CG concentration may be calculated from the Scatchard relationship. When b = n/2, the following substitutions into the Scatchard equation will give the unbound CG concentration:

$$n/2 \cdot 1/[u] = K_a\ (n - n/2)$$

$$[u] = 1/K_a$$

Therefore, the free or unbound CG at n/2 can be determined directly from the slope of the Scatchard plot. The value for b at n/2 can also be obtained directly from the Scatchard relationship. This can be converted to concentration units. If [u] and [b] (for n/2) are summed the total concentration of CG can be obtained which 50% saturates the high affinity site (designated B_{50})

E. <u>Na^+K^+-ATPase/Cardiac Glycoside Interactions -- Binding</u>.

Several groups have shown that there is a parallel relationship between the degree of binding of glycosides and the extent of

inhibition caused by glycosides under like conditions (11,75,77-79). Under conditions similar to those which lead to the phosphorylation of the enzyme (either in the presence of ATP, Mg^{2+} and Na^{+}, or in the presence of Mg^{2+} and P_i) maximal binding is observed for any given set of time and temperature conditions (75,77-80). It has been reported that the number of high affinity binding sites closely approximates the number of sites phosphorylated (77,81). This supports the postulate that a phospho-enzyme is the binding form of $Na^{+}K^{+}$-ATPase. It has been suggested that some binding does occur in the presence of Mg^{2+} only (78), but it is not of the magnitude observed if a phosphate source is present.

An interesting experiment supports the need for a phosphorylated form of the enzyme as the one to which the glycoside binds. Tobin et al. (80) demonstrated that an analogue of ATP, β,γ-methylene ATP, bound to the enzyme in such a fashion as to compete with ATP binding. The analogue is not hydrolyzed by the enzyme. In the presence of β,γ-methylene ATP, Na^{+}, and Mg^{2+} there was only one percent of the ouabain binding observed in the presence of ATP, Mg^{2+}, and Na^{+}. If both ATP and β,γ-methylene ATP were added, there was only 66% of the ouabain binding observed when ATP was present alone.

Although there is much to learn about the form of the enzyme which binds cardiac glycosides, the following summarizes the known ligand effects on this binding. Microsomal eel electroplax $Na^{+}K^{+}$-ATPase binds ouabain in a slow reaction (82) the rate of which can be modified by the following ligands: ATP, orthophosphate, Mg^{2+}, Na^{+} and K^{+}. Two alternate pathways are observed: 1) Na^{+} stimulated, ATP-dependent glycoside binding and 2) Na^{+}-inhibited P_i-dependent

glycoside binding (82). The first pathway can utilize numerous organic phosphate ligands as magnesium complexes (80), while the second is $MgHPO_4$ or Mg^{2+} dependent. The first pathway is inhibited by K^+ while the second is inhibited by Na^+ and K^+ (82-85,79). The free phospho-ligands are found to be inhibitory thus making Mg^{2+} an obligatory ligand (82).

It can be concluded that cardiac glycosides bind preferentially to the E_2-P form of the enzyme (see Figure 1) and may also bind to the E_2 form at a much slower rate (36). It is proposed that the ability of Na^+K^+-ATPase to bind glycosides in the presence of Mg^{2+} and P_i may be related to the E_2 form of the enzyme. This latter postulate arises from the small amount of binding which is observed in the presence of Mg^{2+} but in the absence of a phosphate source (86). The binding may be through the E_2 form of the enzyme or a similar form which is slowly formed in the presence of high concentrations of Mg^{2+}.

As mentioned previously the enzyme system has several features which may be related to its binding of cardiac glycosides. First, Na^+K^+-ATPase is most probably composed of at least two subunits, one a phospholipid containing protein. Second, the system appears to have ligand sites separated by the width of the membrane, i.e. Na^+ and ATP sites on the inside and K^+ and cardiac glycoside sites on the outside of the membrane. Third, although a good relationship between the binding of glycosides and the specific activity of the enzyme has been established in several cases (79,87), binding has been observed under certain conditions that is unrelated to the enzymatic ability to carry out the hydrolysis reaction (88). It is of interest to relate these facts to the observation of Rivas et al. (66) that ^{3}H-ouabain binds to

a hydrophobic protein different from the one which is labelled with ^{32}P in phosphorylation experiments. The latter observation correlates with the RBC membrane work which demonstrates separation of the various ligand sites, some being on the inner surface and some on the outer surface of the membrane.

The role of phospholipids in Na^+K^+-ATPase binding of ouabain has been thoroughly studied by Taniguchi and Iida (81). They observed that ouabain binding rates were unaltered in the presence of either Mg^{2+} and ATP or Mg^{2+}, Na^+ and ATP after phospholipase A treatment. The rates were, however, altered in the presence of Mg^{2+}, K^+ and ATP, Mg^{2+} and P_i, or Mg^{2+} alone after phospholipase A treatment. This suggested that there are at least two binding conformations, one in which phospholipids play a role and the other where they are not involved. As would be expected, the K_d's were unaffected for the first set of conditions and were increased for the second set.

Of great interest is the controversy over the reversibility of the Na^+K^+-ATPase/glycoside complex. Reports of reversibility of binding (50,57,89-95), binding difficult to reverse (96), and virtually irreversible complexes (77,97) are scattered throughout the literature. It would appear that several factors enter into the problem. Species differences appear to be the determining factor, although improper experimental conditions can lead to the false conclusion of irreversibility. The latter situation was pointed out in a paper by Allen, Harris and Schwartz (98). They demonstrated that K^+ would interfere with the reversibility of ^3H-ouabain binding in the presence of Mg^{2+}, ATP and Na^+. This most certainly explained the irreversibility reported by Yoda and Hokin (99) in calf brain dissociation experiments

where they added K^+ to their dissociation medium. Numerous papers have dealt with this problem, but the most complete study was described in two papers by Yoda and coworkers (57,65). Both association and dissociation rate constants were measured for beef brain Na^+K^+-ATPase glycoside interactions. The k_a (for the complex formed in the presence of either ATP, Mg^{2+} and Na^+ or Mg^{2+} and P_i) was dependent upon the nature of the steroid moiety (aglycone). The rank order of the cardiac glycosides was the same when based on either k_a or I_{50}. The k_d for the complex formed in the presence of Mg^{2+} and P_i was dependent upon the nature of the sugar and the temperature but not upon the steroid. Erdmann and Schoner (95) have demonstrated graphically the effect of temperature on the dissociation rates. At 0° ouabain-enzyme complexes of beef kidney, heart and brain as well as dog heart are stable for hours, losing less than 10% bound glycoside in 9 hours. At 37°C 50% of the ouabain has dissociated in less than 2 hours. The same effect has been noted for guinea pig kidney (92) where the binding appeared irreversible at 0°C and was completely reversible at 37°C (see Table 3). The Yoda papers (57,65) did not include bufadienolides or an enzyme which appears, to date, to bind 'irreversibly', Electrophorous electric organ Na^+K^+-ATPase (77). It would seem that the condition of irreversibility of the ATPase/glycoside complex is more a problem of experimental conditions than fact. Since time, temperature, pH, species, glycoside and ligand composition all contribute to the stability of the ATPase/glycoside complex, it is not surprising that confusion prevailed in the past. There is no indication that a covalent bond is formed in the process, and therefore there is no reason to expect that, under proper conditions, an

equilibrium cannot be reached.

Table 3 is a compendium of various equilibrium and rate constants for the glycoside/enzyme complexes of several species. The K_a values were approximated from literature data for comparison with the values given in this dissertation. Of particular interest to this dissertation are the values given for the various guinea pig and rat tissues. It appears that these tissues have Na^+K^+-ATPases that are readily reversible.

In 1969, Allen and Schwartz (75) advanced the idea that the reason the rat was insensitive to cardiac glycosides involved Na^+K^+-ATPases (from rat kidney and heart) that were temperature and time independent; they also suggested that the enzyme/glycoside complex was much less stable than those derived from the enzymes of sensitive species. They claimed maximal binding to rat Na^+K^+-ATPase occurred in less than three minutes. They also reported removal of the ligand by simple washing of the enzyme pellets. Their method for expressing the experimental binding data was the 'direct plot' (see earlier discussion). The use of this method could have led the authors to draw the possibly false conclusion that they were observing a specific binding of glycoside by Na^+K^+-ATPase from the rat heart. It seems highly questionable whether the observed binding was specific or non-specific.

The present study undertook the task of investigating various guinea pig and rat Na^+K^+-ATPases in terms of their ability to bind several glycosides specifically. The Scatchard plot was chosen as the most sensitive method to analyze the experimental binding data. Values for half-saturation of the glycoside high affinity site on

TABLE 3

Binding constants for Na^+K^+-ATPase from various sources

Na^+K^+-ATPase Source	ref.	glycoside[a]	°C	K_a, $10^{-7}M^{-1}$	K_d, 10^8M	k_a $\mu M^{-1}min^{-1}$	assoc $t_{1/2}$ min	k_d $10^{-4}s^{-1}$	dissoc $t_{1/2}$ min
				ATP, Mg^{2+} and Na^+					
guinea pig kidney	(92)	ouabain 2.5×10^{-7} M	37						3
			16						36
			8						120
			0						540
beef brain	(87)	ouabain	37	4.0	2.5				
		1×10^{-7} M							<5[b]
		1×10^{-6} M							<1[b]
rat brain	(78)	ouabain 2×10^{-8} M	37				2.5		
beef kidney	(93)	ouabain	37	9.09	1.1				
beef brain	(65)	digitoxin	25			1.4			
		convallatoxin				1.2			
		cymarin				1.2			
		ouabain				0.64			
beef brain	(57)	ouabain	30					1.17	
human RBC	(100)	ouabain 1.7×10^{-7} M	37	0.72	13.8		30		

TABLE 3 (continued)

Na^+K^+-ATPase Source	ref.	glycoside[a]	°C	$K_a, 10^{-7}M^{-1}$	$K_d, 10^8M$	k_a $\mu M^{-1}min^{-1}$	assoc $t_{1/2}$ min	k_d $10^{-4}s^{-1}$	dissoc $t_{1/2}$ min
				ATP, Mg^{2+} and Na^+					
dog kidney	(101)	ouabain 1×10^{-6} M	37	71.4	0.14		0.3		180
beef brain	(92)	ouabain	37	0.56	18.0				
beef brain	(81)	ouabain	37	0.83	12.0				
rabbit kidney	(94)	ouabain	37						10
dog brain									89
dog heart									45
rat heart									0.05[c]
rat brain									45
guinea pig kidney									2.5
lamb brain	(89)	ouabain 5×10^{-7} M	37	>4.35		2.61		<1.0	>114
electrophorus electricus	(82)	ouabain 1×10^{-5} M	26				t_{max} 15		
calf brain	(83)	ouabain 1×10^{-6} M	37				<1		

TABLE 3 (continued)

Na^+K^+-ATPase Source	ref.	glycoside[a]	°C	$K_a, 10^{-7}M^{-1}$	$K_d, 10^8M$	k_a $\mu M^{-1}min^{-1}$	assoc $t_{1/2}$ min	k_d $10^{-4}s^{-1}$	dissoc $t_{1/2}$ min
				P_i and Mg^{2+}					
beef brain	(81)	ouabain	37	0.91	11.0				
beef kidney	(95)	ouabain	37	21.3	0.47	1.11		0.94	
beef heart				32.2	0.31	2.17		0.99	
beef brain				35.7	0.28	2.78		1.33	
dog heart				23.2	0.43	3.27		2.31	
guinea pig kidney				0.62	16.2	1.05		24.5	
beef brain	(65)	ouabain	25			0.80			
		digitoxin				2.9			
		convallatoxin				1.4			
		cymarin				1.3			
guinea pig kidney	(102)	ouabain	37				1.6		2.5
heart							1.6		2.0
brain							1.6		~20

[a] glycoside concentration given is that used either to saturate enzyme prior to dissociation studies or to estimate association times and K_d values.

[b] estimated from data given.

[c] The value for the rat heart may not be valid since it was given without experimental data to support it.

Na^+K^+-ATPase were compared with the kinetically determined 50% inhibitory values.

EXPERIMENTAL METHODS

A. Chemicals.

The labelled cardiac glycosides convallatoxol-^{3}H (441 mCi/mmole) and cymarol-^{3}H (498 mCi/mmole) were prepared in this laboratory by the reduction of convallatoxin and cymarin with NaB^3H_4 respectively[1]. Ouabain-^{3}H, (1080 mCi/mmole), digoxin-^{3}H (827 mCi/mmole) and digitoxin-^{3}H (533 mCi/mmole) were obtained from New England Nuclear Corporation. Some of the unlabelled glycosides were purchased from Mann Research Laboratories (ouabain), Pfaltz and Bauer, Inc. (digoxin), S. B. Penick and Co. (digitoxin), Fluka AG (cymarin), and L. Light and Co., Ltd. (convallatoxin). Cymarol and convallatoxol were prepared by $NaBH_4$ reduction of cymarin and convallatoxin respectively. Hellebrin was kindly donated by Dr. W. E. Scott of Hoffmann-LaRoche, Inc. Purity of these compounds was established by thin layer chromatography. Tris-ATP was purchased from Sigma Chemical Co. All other chemicals were obtained from commercial sources, were of reagent quality, and were used without further purification. Deionized water that was subsequently glass distilled was used in all aqueous solutions.

B. ATPase Preparation.

In all cases both fresh and frozen (Pel Freeze, Rogers, Arkansas) guinea pig and rat tissues have been used in preparing the enzymes. Equivalent results were obtained with fresh and frozen tissues. Each preparation yielded enough enzyme to carry out a complete set of binding and inhibition experiments. The guinea pig heart enzyme was

studied with respect to variations among identically prepared batches.

Each preparation was carried out entirely at 0-4°C. The volumes for homogenization and resuspension of the pellets are based on weight of the original tissue. The Na^+K^+-specific activities of the enzyme preparations are reported as μmoles P_i released per mg of protein per hour.

Guinea Pig and Rat Ventricle ATPase: A modification of the method of Matsui and Schwartz (105) was used to prepare ventricle Na^+K^+-ATPase. Approximately 30-40 hearts (30-50 g ventricular tissue) were trimmed, chopped and homogenized in a Sorvall Omni-Mixer for 40 sec at medium speed in 9 volumes of 0.25 M sucrose-1.0 mM EDTA (adjusted to pH 7.4 with Tris). The homogenate was filtered through cheese cloth and centrifuged in a Sorvall RC2-B centrifuge at 1020 x g (Sorvall GSA - 2500 rpm) for 10 min. The resulting pellet was resuspended in 8 volumes of 0.25 M sucrose-1.0 mM EDTA (pH 7.4), rehomogenized for 30 sec at low speed, and an appropriate amount of 10% sodium deoxycholate (DOC) was added to yield a 0.1% DOC solution which was then homogenized for 2.5 min at low speed. The DOC treated solution was centrifuged at 23,300 x g (Sorvall GSA - 12,000 rpm) for one hour yielding a pellet which again was treated with 0.1% DOC in 7 volumes of 0.25 M sucrose - 1.0 mM EDTA (pH 7.4). The two supernatants were combined to give a solution which was centrifuged at 68,000 x g (Beckman 42 - 28,500 rpm) in a Beckman L2-65 ultracentrifuge for one hour. The 68,000 x g pellet was resuspended in 1.1 volumes of 1.0 mM EDTA (adjusted to pH 7.4 with Tris) with a Duall glass homogenizer using a Teflon pestle. The final suspensions had protein concentrations of 0.12 -0.15 mg protein/50 μl. The specific activities of the

ventricle Na^+K^+-ATPase preparations were 8-14 and were stable at this level for approximately one month at -20°C.

Guinea Pig Kidney Cortex ATPase: The method of preparation of kidney cortex ATPase was essentially the same as for the ventricle ATPase with the exception that the original centrifugation step was carried out at 4900 x g (Sorvall GSA - 5500 rpm) for 15 min. The final 68,000 x g pellet was resuspended in 1.1 volumes of 1.0 mM EDTA (pH 7.4) yielding a protein concentration of 0.22 mg/50 µl. The enzyme suspensions had Na^+K^+-ATPase specific activities of 8-12. The enzymic activity was stable for at least 6 months.

Guinea Pig and Rat Brain ATPase: A slight modification of the method of Uesugi et al. (106) was used to prepare brain ATPase. The grey matter was aspirated from approximately 35 brains yielding about 100 g of tissue. Cold 0.9% saline was used to wash the aspirated tissue into a collection flask. The suspension of grey matter was centrifuged at 650 x g (Sorvall GSA - 2000 rpm) as it was obtained. The supernatant was reused for collecting additional grey matter. In this way the total volume of saline required was kept to a minimum, 2 l. Finally centrifugation at 1465 x g (Sorvall GSA - 3000 rpm) for 15 min was carried out and the recovered tissue weighed. The resulting pellet was resuspended in 8.5 volumes of 0.32 M sucrose - 1.0 mM EDTA (adjusted to pH 7.4 with Tris), homogenized in a Sorvall Omni-Mixer for 40 sec at medium speed, and centrifuged at 10,400 x g (Sorvall GSA - 8000 rpm) for 20 min. The resulting supernatant was centrifuged in a Beckman 42 or International A-147 rotor at 27,500 x g (19,000 rpm) for 3 hr in either a Beckman L2-65 or an International Model 60B ultracentrifuge. This pellet was resuspended in one volume of

distilled water (pH adjusted to 7.4 with Tris), and treated with an equal volume of 4 M NaI. The 27,500 x g pellet obtained following NaI treatment was washed three times by suspension in 0.8 volume of distilled H_2O, adjusted with Tris to pH 7.4, followed by centrifugation at 27,000 x g (Sorvall SS-34 15,000 rpm). The final suspension of this NaI treated enzyme was in 0.8 volume Tris distilled water (pH 7.4), yielding a protein concentration of 0.15 mg/50 µl with a Na^+K^+-ATPase specific activity of 24-35. The original specific activity was retained for a month and a specific activity of at least 16 was maintained for approximately 3 months.

C. ATPase Assay.

ATPase was assayed by a modification of the method of Matsui and Schwartz (105). The reaction mixture contained a total volume of 0.5 ml -- 50 µl of 20 mM ATP (Tris salt); 50 µl of a combined 0.5 M Tris, 10 mM EDTA, 50 mM $MgCl_2$ solution; 50 µl of a combined 1 M NaCl, 0.2 M KCl solution; made up to 0.45 ml with an appropriate amount of cardiac glycoside solution and/or water. The assay components were preincubated for 5 min at 37°C and 50 µl of the ATPase suspension (0.07 - 0.22 mg protein) was added to start the reaction. The incubation was carried out for 10 min at 37°C on sample and control tubes. The reaction was stopped by the addition of 2.1 ml of cold 5% TCA to the assay mixture. This TCA treated solution was centrifuged in an IEC model UV centrifuge at 1500 rpm for 10 min. A 2.0 ml aliquot of the supernatant was used to measure liberated P_i by the method of Fiske and SubbaRow (107). Na^+K^+-ATPase activity was calculated by the difference in activity with and without complete inhibition by ouabain (5×10^{-4} M). Protein was determined by the method of Lowry et al.

(108).

D. Inhibition Studies.

Experiments to determine 50% inhibition of the enzyme were carried out under assay conditions as stated above with varying amounts of cardiac glycoside (1×10^{-8} to 2×10^{-2} M) and varying amounts of K^+ (0.16 - 20.0 mM). The concentration of glycoside required for 50% inhibition of the Na^+K^+-ATPase activity is referred to as I_{50}.

E. Binding Studies.

Experiments carried out to determine the binding parameters were done in a total volume of 0.25 ml, containing all the substances in identical concentrations as in the assay with the exception of KCl. KCl was omitted in order to obtain optimum binding conditions, and 0.625 mM KCl was used in experiments designed to yield binding parameters which could be compared with inhibition values. Preincubation at 37°C for 5 min preceded the start of the reaction by the addition of 50 µl of the same enzyme suspension. Incubation at 37°C for 10 min was terminated by cooling the tubes on ice. The reaction mixture was centrifuged at 27,000 x g (Sorvall SS-34, 15,000 rpm) for 30 min. This centrifugation was sufficient to sediment essentially all of the ATPase activity. A 200 µl aliquot of the supernatant was pipetted into a counting vial, followed by 0.5 ml of water and 10 ml of Triton-toluene scintillation solution (109). This supernatant aliquot was then counted to determine unbound cardiac glycoside. The excess supernatant was removed from the pellet with a pipet, the tube swabbed and the pellet resuspended in 0.5 ml water. The tubes were kept on ice until this process was complete. A 400 µl aliquot of the resuspended pellet was pipetted into a counting vial, and 0.3 ml of

water was added followed by the Triton-toluene scintillation mixture. This aliquot of the resuspended pellet was counted to yield the value for bound cardiac glycoside. All samples were counted on a Packard Tricarb 3320 and in all cases at least 5000 counts were accumulated. The final form of the data was in pmoles cardiac glycoside/mg protein for the bound portion. The unbound fraction was expressed as molar concentration. A full binding study utilized a concentration range from 1×10^{-8} to 3×10^{-5} M cardiac glycoside with a specific activity range from 2.5 to 666 mCi/mmole (see Table 4). Several experiments employing 1×10^{-9} M cardiac glycoside solutions did not yield consistently higher bound/unbound cardiac glycoside ratios. From this it was assumed that a concentration of 1×10^{-8} M was closely approaching the limiting value of nK_a (see discussion of the Scatchard plot), and therefore concentrations of less than 1×10^{-8} M were not regularly used in binding studies.

The experimental binding data in this dissertation were treated so as to yield values which could be plotted by the Scatchard method (see earlier discussion). The radioactivity of the supernatant was used to calculate pmoles of cardiac glycoside unbound, and the pellet values to calculate pmoles cardiac glycoside bound. From these results the bound to unbound ratio was calculated. An extrapolation was carried out for each plot to yield the binding parameters n, nK_a and K_a. The n values are given in units of pmoles CG bound/mg protein; the K_a values are expressed as reciprocal molar concentrations.

The binding experiments were carried out in total volumes of 0.25 ml whereas the inhibition studies were in 0.5 ml. The amount of protein was the same in both cases. Therefore, the B_{50} values have been

TABLE 4

Specific activities of glycosides used in binding studies

The tritiated glycosides were diluted with appropriate amounts of the corresponding unlabeled compounds to yield the indicated specific activities, which were suitable for accurate estimates of binding at the various stated concentrations.

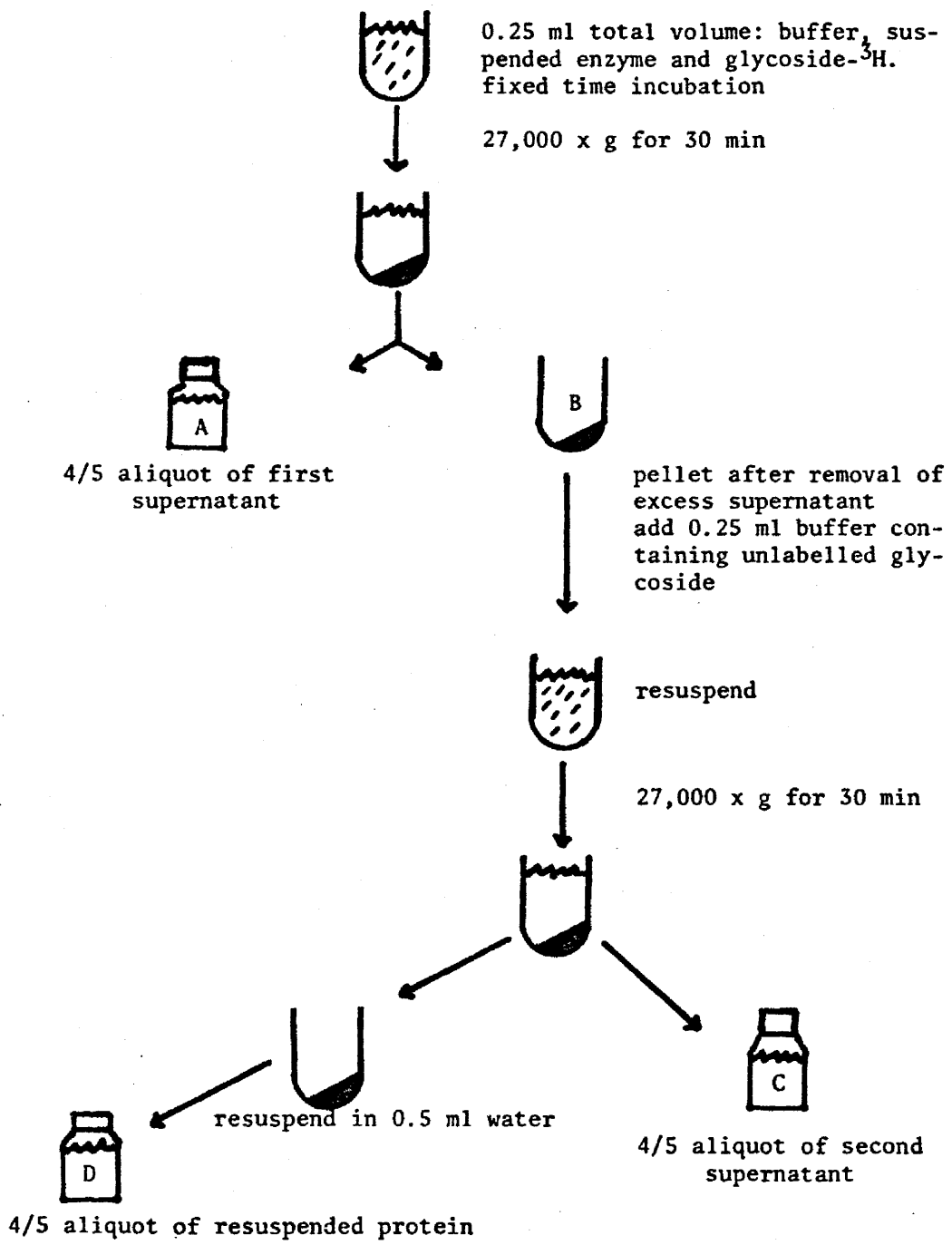
Molar Concentration of Glycoside	Specific Activity in mCi/mmole				
	Convallatoxol	Cymarol	Digoxin	Digitoxin	Ouabain
1 & 3 x 10^{-8}	441.0	401.0	665.0	666.0	665.0
1 & 3 x 10^{-7}	263.0	211.0	33.2	33.2	33.3
1 & 3 x 10^{-6}	26.3	21.1	3.3	3.3	3.3
1 & 3 x 10^{-5}	19.8	15.8	2.5	2.5	2.5

calculated to yield the original concentration of cardiac glycoside required to give half saturation of the binding site had 0.5 ml, rather than 0.25 ml, been used.

To establish that a condition of equilibrium existed between glycoside-^{3}H and ATPase, incubations were carried out under standard binding conditions plus a second incubation that employed a replacement of glycoside-^{3}H with cold glycoside. A fixed amount of enzyme and tritiated cardiac glycoside (1×10^{-8} M) were allowed to interact at 37°C for a fixed time interval. This incubation was terminated by cooling on ice as usual. The samples were then centrifuged as usual. The supernatant-A (see Figure 5) was totally removed and an aliquot counted. The pellet-B was resuspended in the same reaction medium with the substitution of 1×10^{-5} M cold glycoside for the glycoside-^{3}H. The resuspended pellet including any enzyme-glycoside-^{3}H complex was incubated for varying time periods (2.5-60 minutes) at 37°C and the incubation was again terminated, the contents centrifuged, and the protein and supernatant assayed for tritium. Counts from the pellet-D plus the second supernatant-C represent those counts that were present in the original pellet-B. The data therefore contains an expression of the original equilibrium (original bound = C + D; original unbound = A; and the original b/u ratio = $\frac{C + D}{A}$ and the degree of dissociation (bound = D; unbound = C; and b/u = D/C) at various times.

Figure 5. A protocol for the binding studies used to determine dissociation data.

Samples A, C, D were counted to yield the experimental binding data; C and D are representative of the unbound and bound for the dissociation phase of the experiment.


0.25 ml total volume: buffer, suspended enzyme and glycoside-^{3}H. fixed time incubation
27,000 x g for 30 min
A
4/5 aliquot of first supernatant
B
pellet after removal of excess supernatant
add 0.25 ml buffer containing unlabelled glycoside
resuspend
27,000 x g for 30 min
resuspend in 0.5 ml water
C
4/5 aliquot of second supernatant
D
4/5 aliquot of resuspended protein

RESULTS

A. Guinea Pig Brain, Kidney and Heart Na^+K^+-ATPase.

ATPase Assay: The assay of Na^+K^+-ATPase consists of two steps, the first of which is the enzymatic cleavage of the γ-phosphate from ATP under the appropriate conditions (see Experimental Methods) either in the presence or absence of 1×10^{-3} M ouabain. In the absence of ouabain both Mg^{2+} and Na^+K^+-dependent ATPase activities are expressed, yielding a total production of P_i that is higher than the value obtained in the presence of ouabain. In the latter case the Na^+K^+-ATPase activity is inhibited, and therefore the production of P_i under these conditions is an expression of Mg^{2+}-dependent ATPase. The Na^+K^+-ATPase activity is obtained as the difference between the two values. The second step of the assay is a colorimetric determination of the P_i produced in the enzymatic incubation. Typical data for a K_2HPO_4 standard curve are shown in Table 5. From such data was obtained the value A_{660} units/μmole P_i that was used to convert absorbance readings into units of enzyme activity. This value is given in each assay or inhibition table (see Appendix Tables 1-10). Also included in Table 5 are example enzyme assay data like those used to calculate the Na^+K^+-ATPase specific activities cited for each table of assay or inhibition data.

Each guinea pig Na^+K^+-ATPase displayed a linear production of P_i over the time period in which all assays were carried out. There was also, a linear increase in the rate of production of P_i with a

TABLE 5

An example set of data for the enzymatic assay of Na^+K^+-ATPase, including the standard curve for phosphate determination

Guinea pig heart Na^+K^+-ATPase (4.4 mg protein/ml) was assayed under the conditions described in Experimental Methods at a K^+ concentration of 0.625 mM for 10 minutes; the value obtained from the phosphate standard curve, 1.27 A_{660} units/µmole P_i, was used to convert the assay absorbance values into activity values.

Phosphate Determination Standard Curve

µmole KH_2PO_4	A_{660}
0.25	.338
0.25	.361
0.50	.66
0.50	.64
1.00	1.22
1.00	1.22

1.27 A_{660} units/µmole P_i

Enzyme Assay at 0.625 mM K^+

Enzyme	A_{660}	Activity[a]
1	.164	22.4
2	.152	20.7
3	.151	20.6
4	.163	22.2
Enzyme + Ouabain		
1	.085	11.6
2	.094	12.8
3	.075	10.2

[a] Enzyme - (Enzyme + Ouabain) = Na^+K^+-ATPase Activity

(10.0 µmoles P_i/mg Pro/hr)

progressive increase in enzyme concentration (see Appendix Table 1).

ATPase Activity as a Function of K^+ Concentration: Figure 6 demonstrates that the activity of preparations from all three guinea pig organs varies with K^+ concentration. It can be seen that 0.625 mM K^+ gives adequate ATPase activity to allow activity measurements for all three ATPase preparations. This concentration of K^+ is also sufficiently low for binding studies, as discussed below. Although heart and kidney preparations have approximately the same specific activity, the activity measured for heart ATPase at 0.625 mM K^+ is only 45% of its maximal activity whereas kidney is at least 95% of its maximal activity.

ATPase Inhibition as a Function of K^+ Concentration: Figure 7 demonstrates that the effective concentration of CG required to cause 50% inhibition occurs at a much lower glycoside concentration in the presence of 0.625 mM K^+ than in the presence of 20.0 mM K^+. This dramatic difference (Table 6) is evident in the inhibition studies of each of the enzyme preparations, i.e. guinea pig heart, kidney, and brain (data for the determination of I_{50} values, Appendix Tables 5-9). Differences are about 30-200 fold for brain, 20-80 fold for kidney and 25-250 fold for heart ATPase.

Na^+K^+-ATPase Relationship to High Affinity Binding: Full binding studies on all the cardiac glycosides yield high affinity and low affinity portions of the curve when a Scatchard plot is applied to the binding data (all data cited in Appendix Tables 10-30). The high affinity region is that portion of the curve which encompasses data points described by relatively small amounts of ligand bound at a high b/u ratio, while the low affinity region includes data points

Figure 6. Effect of varying K^+ concentration on Na^+K^+-ATPase activity from three guinea pig organs.

The assays were carried out as described under Experimental Methods except the K^+ concentrations were varied as indicated. Specific activities (20 mM K^+) of the enzyme preparations were: heart - 10.0 (✿), brain - 30.1 (●), and kidney - 12.0 (■) (see Appendix Tables 2-4).

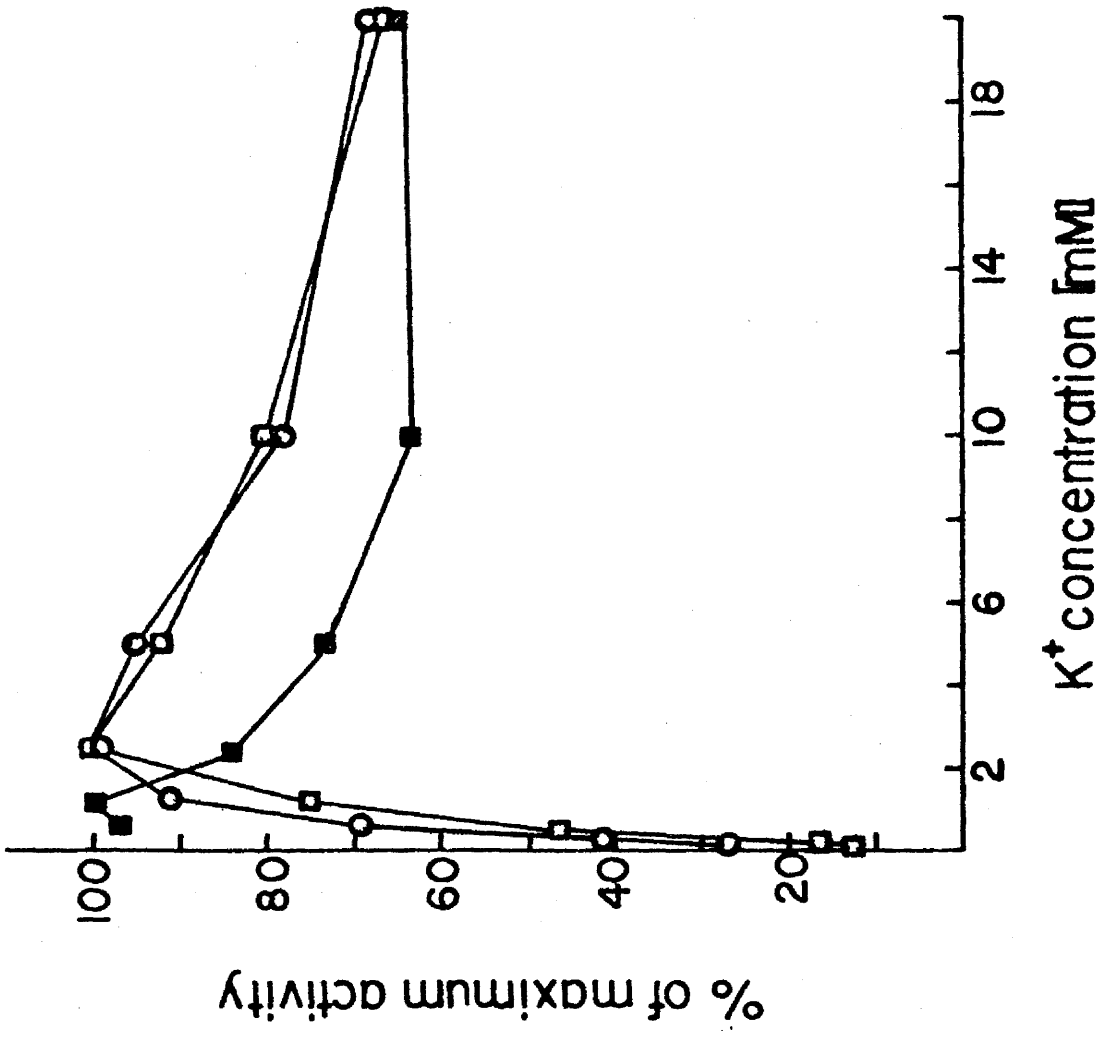

% of maximum activity
100
80
60
40
20
2
6
10
14
18
K+ concentration (mM)

Figure 7. Effect of K^+ concentration on I_{50} determination with guinea pig kidney Na^+K^+-ATPase.

Assays of ATPase activity were carried out as described under Experimental Methods in the presence of either 0.625 mM K^+ (●) or 20.0 mM K^+ (⊙). The dashed lines (---) show the I_{50} values (see Appendix Tables 5-9).

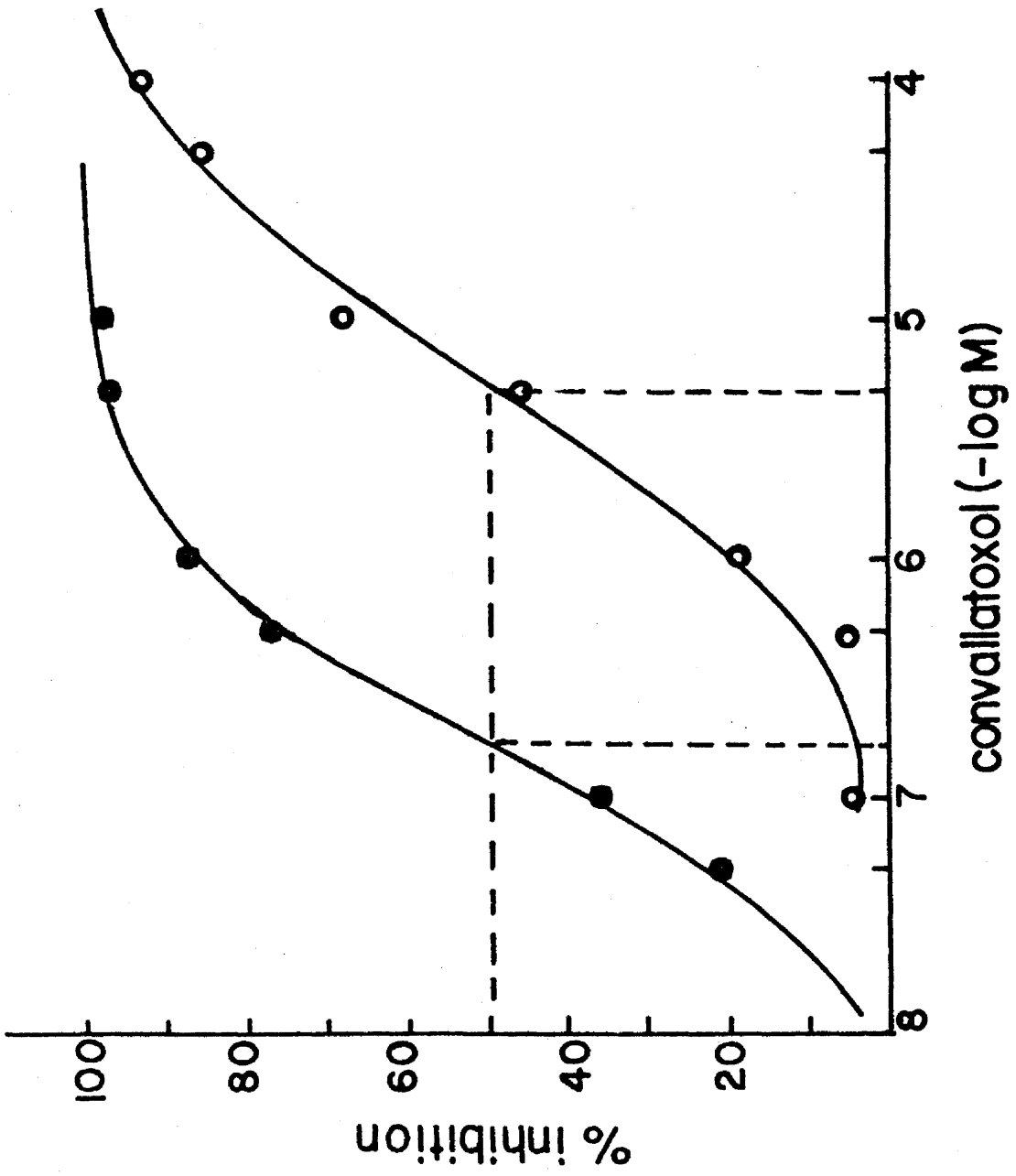

% inhibition
100
80
60
40
20
8
7
6
5
4
convallatoxol (-log M)

TABLE 6

Inhibition studies: I_{50} determination at two K^+ concentrations

Na^+K^+-ATPase from guinea pig brain (specific activity 30.1), kidney (specific activity 12.0), and heart (specific activity 10.0) were assayed as described under Experimental Methods with concentrations of glycosides varying from 1×10^{-8} to 1×10^{-4} M in the presence of either 0.625 mM K^+ or 20.0 mM K^+. The I_{50} values were estimated graphically as indicated in Figure 7.

	I_{50} (μM)					
	G.P. Brain[b]		G.P. Kidney[c]		G.P. Heart[d]	
	0.625[a]	20.0	0.625	20.0	0.625	20.0
Digitoxin	0.03	1.9	0.13	4.8	0.13	3.0
Cymarol	0.04	3.7	0.22	8.4	0.06	16.0
Convallatoxol	0.09	2.8	0.16	5.3	0.12	24.0
Digoxin	0.03	7.1	0.27	22.0	0.12	24.0
Ouabain	0.12	7.1	0.57	13.0	0.12	23.0
Hellebrin	0.02	1.2	0.13	3.0	0.03	5.0

[a] Potassium chloride concentration, (mM).

[b] see Appendix Tables 6-7.

[c] see Appendix Tables 8-9.

d see Appendix Table 5.

described by low b/u ratios and relatively large amounts of ligand bound (Figure 4). In view of the dependence of binding of the tritiated glycoside to Na^+K^+-ATPase upon the phosphorylation of the enzyme by ATP and the further dependence of phosphorylation on the presence of Mg^{2+} and Na^+ (7,36-41), it can be predicted that specific binding of the tritiated glycoside to Na^+K^+-ATPase will not occur in the absence of ATP, Mg^{2+}, or Na^+. Furthermore, high affinity binding cannot be detected in the presence of high concentrations of K^+, since this ion causes the breakdown of the phosphorylated form of the enzyme (7). Experiments were done to relate Na^+K^+-ATPase activity to the high affinity portion of the Scatchard plot. Maximum high affinity binding of tritiated cardiac glycoside to enzyme occurred in the presence of ATP, Mg^{2+}, and Na^+. Omission of any one of these greatly reduced the slope of the high affinity binding portion of the curve (see Appendix Table 10).

Binding as a Function of Enzyme Concentration: Using a fixed glycoside concentration and increasing amounts of protein (Table 7), several observations can be made on the binding of glycosides by Na^+K^+-ATPase. It binds convallatoxol-^3H with a higher affinity than it binds either digoxin-^3H or ouabain-^3H, a finding consistent with the Scatchard data. At low protein concentrations the b/u ratio increases linearly, while at higher protein concentrations the ratio asymptotically approaches a limiting value of infinity. This correlates with the approach to infinity of the b/u ratio along the y-axis of a Scatchard plot as the glycoside concentration decreases at constant protein concentration.

TABLE 7

Experimental binding data for guinea pig heart Na^+K^+-ATPase as a function of protein concentration

The binding studies were carried out with guinea pig heart Na^+K^+-ATPase (specific activity 8.5; 2.3 mg protein/ml) and either 1 x 10^{-8} M convallatoxol-^3H, digoxin-^3H or ouabain-^3H as described under Experimental Methods at the specified enzyme concentrations (see Appendix Table 11).

µl Enzyme Solution	Expt. 1		Expt. 2		Expt. 3	
	bound	b/u	bound	b/u	bound	b/u
Convallatoxol						
25	2.21	0.219	1.91	0.216	2.6	1.01
	2.81	0.308	2.39	0.269	2.3	0.88
50	5.45	0.658	4.68	0.609	5.0	2.07
	5.36	0.612	5.22	0.656	4.9	2.09
100	7.95	1.12	7.65	1.22	7.6	3.79
	7.88	1.10	7.40	1.18	7.9	4.12
150	9.74	1.53	9.38	1.68	10.4	6.04
	10.0	1.57	8.96	1.57	9.7	5.64
Digoxin						
25	0.70	0.078	1.02	0.131	0.9	0.38
	0.72	0.080	0.96	0.119	0.9	0.38
50	1.75	0.200	1.96	0.262	2.1	0.97
	1.90	0.229	1.99	0.262	1.7	0.76
100	3.41	0.418	3.57	0.554	3.6	1.69
	3.73	0.496	3.22	0.485	3.2	1.56
150	4.90	0.692	5.22	0.821	4.5	2.27
	4.55	0.643	4.84	0.752	4.3	2.20
Ouabain						
25	1.16	0.116	1.05	0.122	1.1	0.42
	1.17	0.120	1.01	0.117	1.1	0.45
50	2.57	0.260	2.28	0.260	2.3	0.90
	2.41	0.252	2.14	0.254	2.2	0.87
100	3.89	0.229	4.01	0.530	3.5	1.54
	3.82	0.220	4.04	0.555	3.9	1.79
150	5.60	0.376	5.09	0.766	5.2	2.45
	5.08	0.353	4.97	0.718	5.2	2.45

ATPase Binding as a Function of K^+ Concentration: A sharp decline in the b/u ratio at a fixed total concentration of glycoside occurs with increasing concentration of K^+ for all three enzyme preparations (see Appendix Tables 12-13). Relatively high b/u ratios are still obtained at 0.625 mM K^+; this concentration can therefore be used satisfactorily for B_{50} determinations.

Figure 8 demonstrates the effect of increasing K^+ concentration on the Scatchard plot of the high affinity binding curve of guinea pig heart ATPase with convallatoxol-^3H (see Appendix Tables 12). It is apparent from this study and similar experiments on brain and kidney ATPase that increasing K^+ (Appendix Table 13) interferes with binding to all three enzymes. With increases in K^+ concentration, the affinity constant and the number of high affinity binding sites are reduced. When the B_{50} values are calculated, there is a corresponding increase in the cardiac glycoside concentration necessary to obtain 50% saturation of the high affinity site (Table 8) as the K^+ concentration is increased. At high concentrations of K^+, the B_{50} values cannot be estimated since any specific binding would occur only at cardiac glycoside concentrations in the range where non-specific binding also occurs.

Binding Parameters of Three Tissue ATPase Preparations at 0.0 and 0.625 mM K^+ with Various Cardiac Glycosides: The B_{50} values (Table 9) for all three preparations show no great differences when studied under optimum conditions in the absence of K^+ and in the presence of 0.625 mM K^+. The latter concentration of K^+ supports ATPase activity adequate for estimation of the I_{50}. Although theoretically the B_{50} values at 0.0 K^+ should be lower than at 0.625 mM K^+,

Figure 8. Effect of various K^+ concentrations on the high affinity binding of convallatoxol-^3H to guinea pig heart Na^+K^+-ATPase.

Binding studies were carried out with guinea pig Na^+K^+-ATPase (specific activity 9.5) and convallatoxol-^3H as described under Experimental Methods in the presence of various KCl concentrations (■ 0 K^+, ● 0.155 mM K^+, ■ 0.625 mM K^+, ○ 20.0 mM K^+) (see Appendix Table 12).

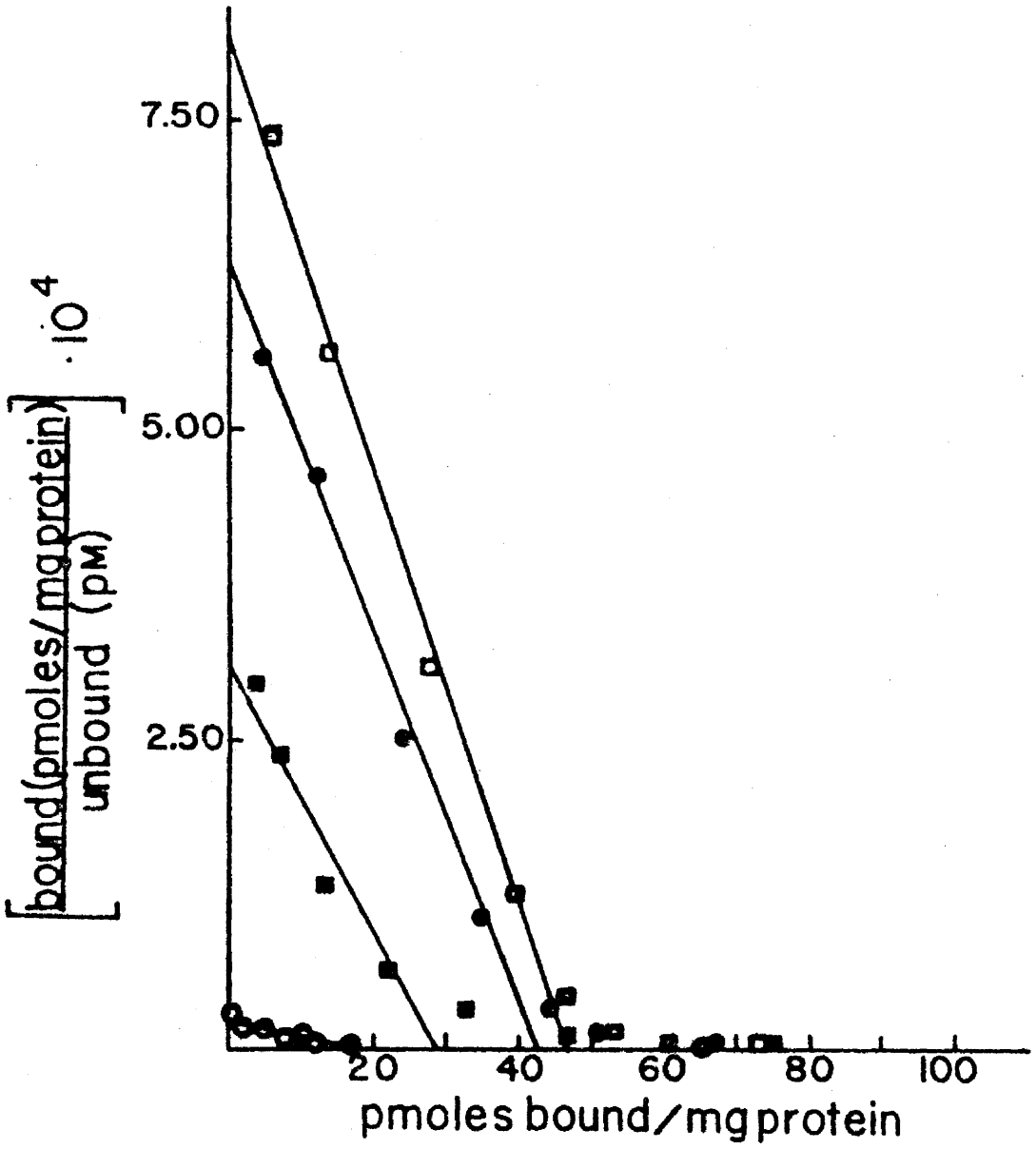

7.50
5.00
2.50
20
40
60
80
100
pmoles bound/mg protein
[bound(pmoles/mg protein) / unbound (pM)] ·10^4

TABLE 8

K_a and B_{50} values as a function of K^+ concentration

The K_a and B_{50} values of guinea pig brain ATPase (specific activity 20.0) with convallatoxol-^3H were calculated from Scatchard plots of data obtained at the stated K^+ concentrations (see Appendix Table 14).

$[K^+]$	K_a, 10^{-7} M^{-1}	B_{50}
mM		μM
0.0	3.55	0.039
0.625	2.57	0.050
1.25	2.13	0.057
2.50	1.42	0.080

TABLE 9

Binding parameters for various glycosides in three guinea pig tissues at 0 K^+ and 0.625 mM K^+

The binding parameters were determined as described in Experimental Methods for each of the tritiated glycosides with brain (specific activity 21.9), kidney (specific activity 11.5), and heart (specific activity 10.2) Na^+K^+-ATPase in the absence of K^+ and the presence of 0.625 mM K^+. Each value in the table is the mean from several experiments except those with heart and kidney Na^+K^+-ATPase, under conditions of 0.625 mM K^+, which are from a single experiment.

	0 K^+			0.625 mM K^+		
Cardiac Glycoside	B_{50}[a]	n[b]	$K_a, 10^{-7}$ M^{-1}	B_{50}[a]	n[b]	$K_a, 10^{-7}$ M^{-1}
Brain:[c]						
Digitoxin	-	-	-	0.04	146	4.76
Cymarol	0.04	140	7.22	0.04	133	3.75
Convallatoxol	0.03	130	12.49	0.03	130	5.64
Digoxin	0.08	147	1.95	0.08	125	1.44
Ouabain	0.09	165	1.82	0.12	157	1.00
Kidney:[d]						
Digitoxin	0.09	83	1.50	0.12	69	0.97
Cymarol	0.10	105	1.28	0.20	93	0.55
Convallatoxol	0.10	101	1.24	0.22	100	0.50
Digoxin	0.18	76	0.64	0.36	80	0.30
Ouabain	0.21	109	0.54	0.61	108	0.17

TABLE 9 (continued)

Cardiac Glycoside	0 K^+			0.625 mM K^+		
	B_{50}	n	K_a, 10^{-7} M^{-1}	B_{50}	n	K_a, 10^{-7} M^{-1}
Heart:[e]						
Digitoxin	0.04	35	2.68	0.06	35	1.95
Cymarol	0.06	41	1.98	0.05	29	2.00
Convallatoxol	0.08	48	1.40	0.09	39	1.18
Digoxin	0.13	37	0.83	0.10	25	1.08
Ouabain	0.17	50	0.61	0.16	31	0.65

[a] The B_{50} values are micromolar.

[b] The values of n are expressed as pmoles cardiac glycoside bound/mg protein

[c] see Appendix Tables 15-20.

[d] see Appendix Tables 21-23.

[e] see Appendix Tables 24-29.

this was not consistently observed except in the case of the kidney. This may reflect a variation in purity of the preparations as well as error in the measurement, since the measurements were made on different preparations at different times. The n values (amount bound per mg protein at saturation of the high affinity site) tend to reflect the differences in Na^+K^+-ATPase specific activities among the various tissues, although there is not a constant relationship. As the specific activity decreases with age for a given preparation the n value decreases at a slower rate (Table 10).

The differences dependent upon K^+ concentration observed in brain and kidney K_a values are greater than those differences observed for heart (Table 9). This indicates a greater effect of K^+ on the apparent binding affinity of brain and kidney ATPase for cardiac glycosides. There is a consistent trend toward digoxin and ouabain being bound less tightly as evidenced by the lower K_a values and higher B_{50} values for these two compounds. The data for digitoxin binding to brain ATPase in the absence of K^+ were much more variable than those for the other glycosides and are therefore not included in Table 9 (see Appendix Tables 14-19).

Comparison of I_{50} and B_{50} Values at 0.625 mM K^+ from 3 Tissue ATPase Preparations: Optimum binding conditions (i.e. without K^+) give B_{50} values that show 50-300 fold differences from the I_{50} values determined under normal assay conditions (20.0 mM K^+) (Tables 6 and 9). If both binding and inhibition are carried out at 0.625 mM K^+, the B_{50} and I_{50} values (Table 11) then fall within the same range. The agreement between individual values is reasonably good when the possible sources of error are considered. Both ouabain and digoxin

TABLE 10

Changes in specific activity and n values of guinea pig heart ATPase with time

Assays and binding studies with convallatoxol were carried out on guinea pig heart ATPase at various times after its preparation. The enzyme was stored at -20°C.

Age of preparation in days	Specific activity	n
	μmole P_i/ mg protein/hr	pmoles bound/ mg protein
1	7.1	53
6	7.2	55
13	5.7	44
36	2.0	45

TABLE 11

Comparison of I_{50} and B_{50} values at 0.625 mM K^+ for Na^+K^+-ATPase from three guinea pig tissues

This table compares selected data from Tables 6 and 9.

Glycoside	G.P. Brain		G.P. Kidney		G.P. Heart	
	I_{50}	B_{50}	I_{50}	B_{50}	I_{50}	B_{50}
	µM	µM	µM	µM	µM	µM
Digitoxin	0.03	0.04	0.13	0.12	0.13	0.06
Cymarol	0.04	0.04	0.22	0.20	0.06	0.05
Convallatoxol	0.09	0.03	0.16	0.22	0.12	0.09
Digoxin	0.03	0.08	0.27	0.36	0.12	0.10
Ouabain	0.12	0.12	0.57	0.61	0.12	0.16

appear to be bound less tightly by brain and kidney Na^+K^+-ATPase when both I_{50} and B_{50} values are considered. The binding of ouabain and digoxin by heart Na^+K^+-ATPase appears to be comparable to that of the other three glycosides. The overall range of glycoside concentration required to half-saturate or half-inhibit the enzyme is 3-61 x 10^{-8} M. Most of the variation is in kidney Na^+K^+-ATPase interactions with the glycosides. If kidney is excluded, the range narrows considerably, 3-16 x 10^{-8} M.

ATPase Binding of Cardiac Glycosides in the Presence of P_i and Mg^{2+}: One millimolar P_i used in the presence of Mg^{2+} will support binding which yields Scatchard plots similar to those for binding in the presence of ATP, Mg^{2+} and Na^+ (see Appendix Table 29). If either 4.0 mM K^+ or 100 mM Na^+ is included, the maximal b/u ratio is drastically decreased (Table 12). This inhibitory effect has been seen with as little as 2 mM Na^+. When the incubation is carried out in the presence of P_i alone, or with P_i and Na^+, there is no observable high affinity binding (i.e. the b/u ratio is very low).

B. Rat Brain Na^+K^+-ATPase.

Rat Brain Assay: The enzyme was assayed over a thirty minute period (Figure 9) and displayed a linear production of P_i over the time span in which all assay and inhibition studies were carried out (10 minutes). There also was a linear increase in the production of P_i with increasing enzyme concentration (Figure 10). When enzyme activity was studied (at a fixed time interval and at fixed reactant concentrations) as a function of K^+ concentration (Figure 11), the maximum Na^+K^+-ATPase activity (as well as total ATPase activity) was seen at 2.5 mM K^+, with relatively high activity maintained over a K^+

TABLE 12

The effect of ionic variations on the binding of glycoside to guinea pig Na^+K^+-ATPase in the presence of P_i

All tubes contained 50 µl enzyme suspension and 1 x 10^{-8} M convallatoxol-^3H and, where indicated, 2 mM ATP(Tris), 1 mM H_3PO_4, 5 mM $MgCl_2$, 100 mM NaCl, 4 mM KCl in 0.25 ml at pH 7.4. The binding experiments were carried out as described under Experimental Methods.

	Relative b/u	
	Brain	Kidney
ATP, Mg^{2+}, Na^+	100	100
P_i	1	-
P_i, Na^+	<1	-
P_i, Mg^{2+}	76	109
P_i, Mg^{2+}, Na^+	1	-
P_i, Mg^{2+}, Na^+, K^+	<1	1
P_i, Mg^{2+}, K^+	1	-

Figure 9. Hydrolysis of ATP by rat brain Na^+K^+-ATPase as a function of time.

The assay of rat brain Na^+K^+-ATPase (specific activity 33.2) was carried out as described under Experimental Methods (see Appendix Table 31).

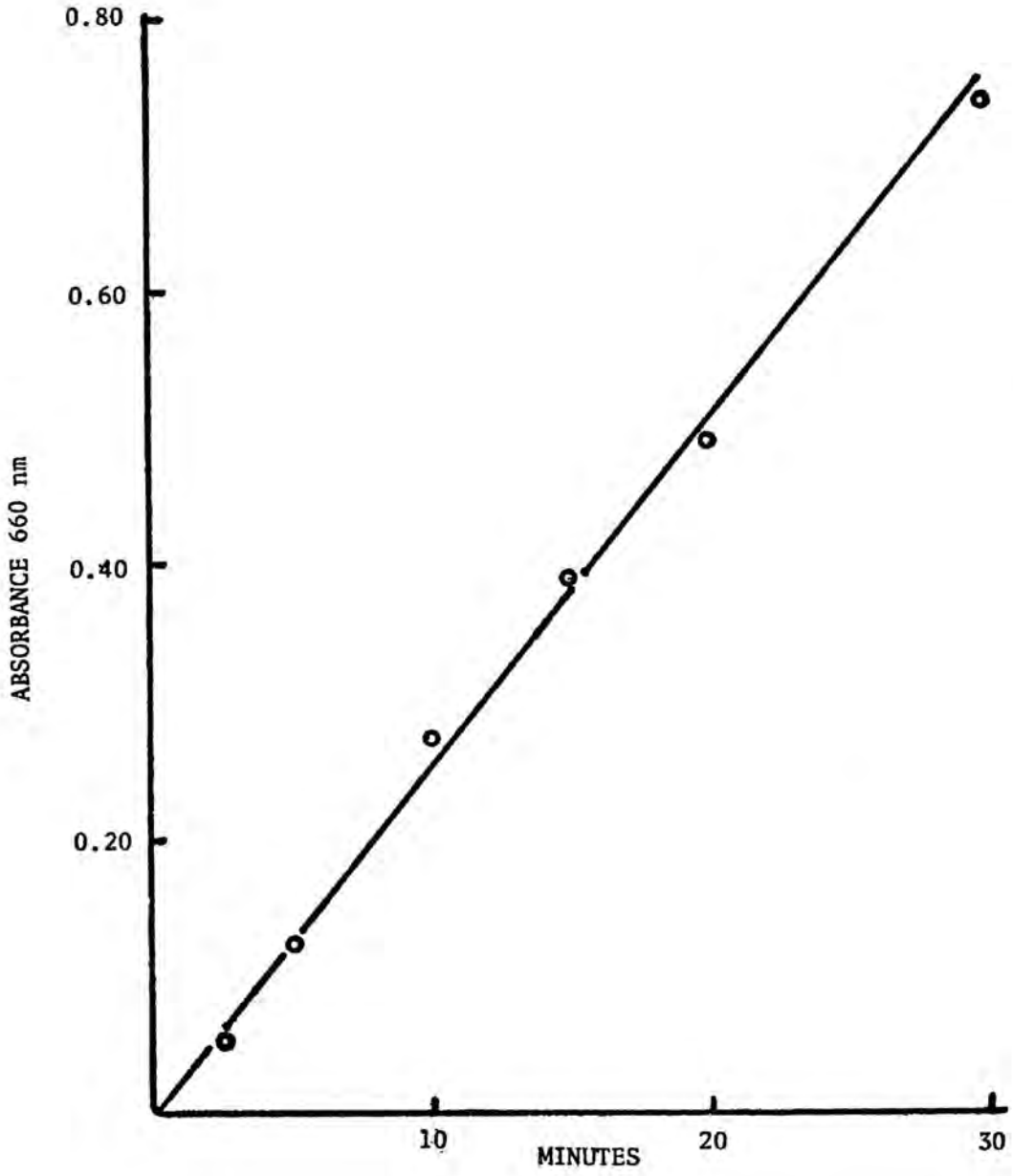

0.80
0.60
0.40
0.20
ABSORBANCE 660 nm
10
20
30
MINUTES

Figure 10. Rat brain Na^+K^+-ATPase activity as a function of enzyme concentration.

The assay of rat brain Na^+K^+-ATPase (specific activity 24.0; 0.90 mg protein/ml) was carried out as described under Experimental Methods with the specified volumes of enzyme solution (see Appendix Table 32).

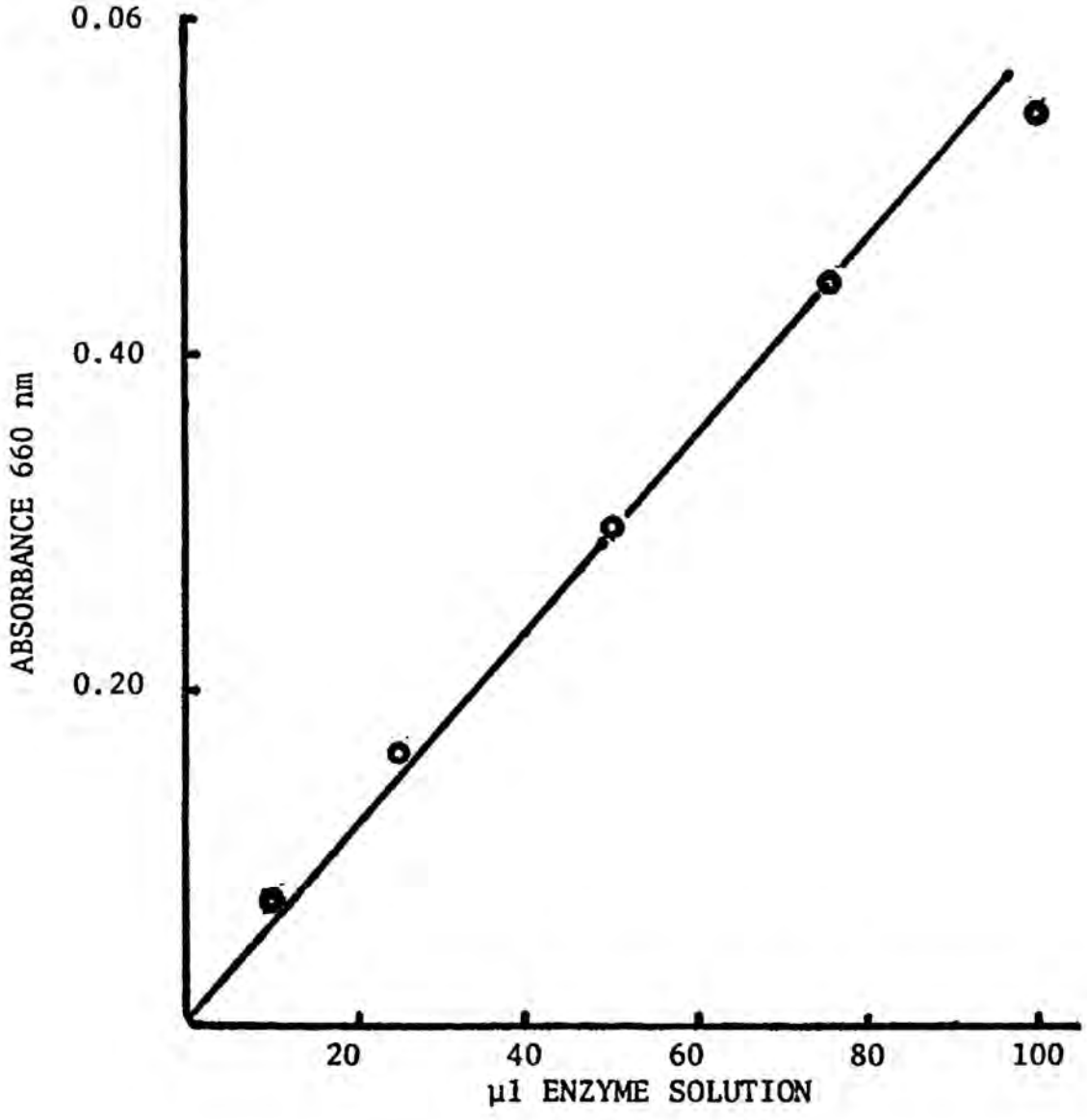
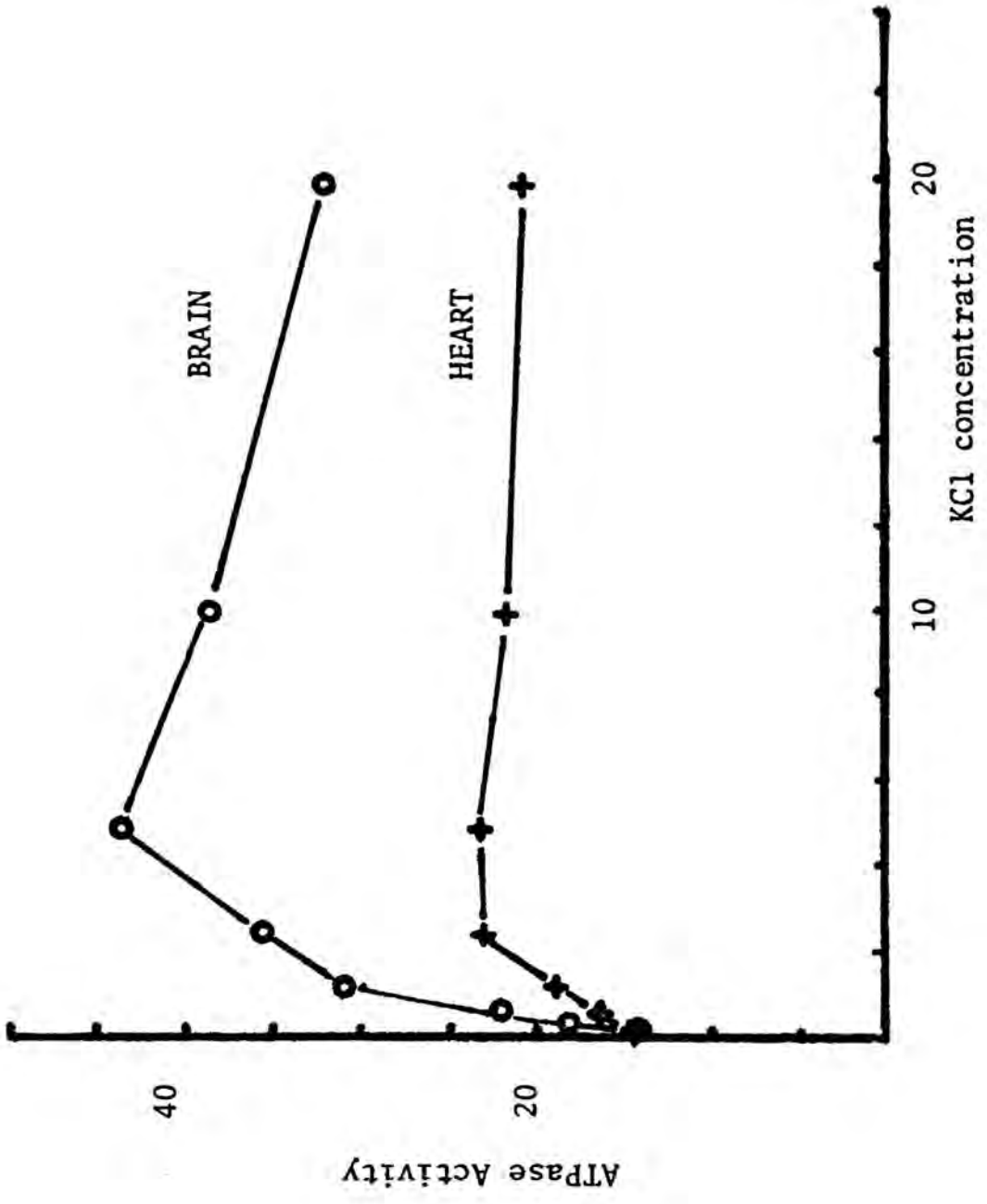

0.06
0.40
0.20
ABSORBANCE 660 nm
20
40
60
80
100
µl ENZYME SOLUTION

Figure 11. The effect of varying K^+ concentration on two rat Na^+K^+-ATPases.

The assays were carried out as described under Experimental Methods except the K^+ concentrations were varied as indicated. Specific activities were: brain - 30.3 (●), heart - 13.2 (+) (see Appendix Table 33).


ATPase Activity
40
20
BRAIN
HEART
10
20
KCl concentration

concentration range of 1-20 mM.

Inhibition of Rat Brain Na^+K^+-ATPase by Various Cardiac Glycosides at 0.625 and 20.0 mM K^+: The I_{50} values (Table 13) for rat brain are in the micromolar range with little difference found between those at low K^+ concentration (0.625 mM) and those at high K^+ concentration (20 mM). It can also be seen from Table 13 that there is little difference among I_{50} values for the various glycosides. While digoxin and ouabain appear to be required in higher concentrations than the others to effect 50% inhibition at 20 mM K^+, this does not seem to be the case at 0.625 mM K^+. These I_{50} values at 0.625 mM are 60-100 fold greater than those observed with the guinea pig brain enzyme (see Table 6) and 10-30 fold greater than for guinea pig kidney or heart enzyme.

Binding Parameters of Rat Brain Na^+K^+-ATPase for Various Cardiac Glycosides: From Table 14 it can be seen that the B_{50} values fall in the 1×10^{-8} M range. The B_{50} values (Table 14, Preparation 1) for digitoxin, cymarol and convallatoxol in the presence of 0.625 mM K^+ fall in the range of $2.2\text{-}3.6 \times 10^{-8}$ M while those for digoxin and ouabain are considerably higher 8.4 and 7.3×10^{-8} M. These values for B_{50} are slightly higher than the B_{50} values at 0 K^+ (Table 14). Preparation 2 demonstrates that a decrease in affinity and increase in n markedly affect B_{50} values. These data are similar to the data obtained for guinea pig brain (Table 9) not only in terms of relative order but also in magnitude. The K_a values (Table 14-Preparation 1) for digitoxin, cymarol and convallatoxol in the presence of 0.625 mM K^+ fall within the range of $4.8\text{-}7.6 \times 10^7 M^{-1}$, while digoxin and ouabain have K_a values of 1.4 and $1.6 \times 10^7 M^{-1}$ respectively. As would be

TABLE 13

Rat Brain Inhibition Studies:
I_{50} determination at two K^+ concentrations

ATPase from rat brain (specific activity 16.0-24.0) was assayed as described under Experimental Methods with concentrations of glycosides varying from 1×10^{-8} M to 5×10^{-4} M in the presence of either 0.625 mM K^+ or 20 mM K^+. The I_{50} values were estimated graphically as indicated in Figure 7 (see Appendix Table 34).

	I_{50} (μM)	
	0.625*	20.0*
Digitoxin	2.1	1.6
Cymarol	3.2	1.1
Convallatoxol	1.7	2.2
Digoxin	1.6	3.2
Ouabain	3.2	3.8
Hellebrin	4.3	1.7

* Potassium chloride concentration, mM

TABLE 14

Binding parameters for various glycosides in rat brain at 0 K^+ and 0.625 mM K^+

The binding parameters were determined as described in Experimental Methods for each of the tritiated glycosides with rat brain Na^+K^+-ATPase (specific activity 30.3, preparation 1, and 37.0, preparation 2) in the absence of K^+ and in the presence of 0.625 mM K^+ (see Appendix Tables 35-37).

Cardiac Glycoside	0 K^+			0.625 mM K^+		
	B_{50} [a]	n [b]	Ka, $10^{-7}M^{-1}$	B_{50} [a]	n [b]	K_a, $10^{-7}M^{-1}$
			Preparation 1			
Digitoxin	0.019	79	6.5	0.036	80	4.8
Cymarol	0.019	64	6.3	0.028	57	5.4
Convallatoxol	0.017	81	7.7	0.022	71	7.6
Digoxin	0.090	83	1.2	0.084	64	1.4
Ouabain	0.051	75	2.1	0.073	66	1.6
			Preparation 2			
Digitoxin	0.064	192	3.0			
Cymarol	0.044	139	4.6			
Convallatoxol	0.034	123	7.1			
Digoxin	0.092	125	1.4			
Ouabain	0.093	135	1.4			

[a] the B_{50} values are given in micromoles/liter.

[b] the n values are expressed as pmoles cardiac glycoside per mg protein.

expected, the K_a values in the presence of 0.625 mM K^+ are slightly lower than those obtained in the absence of K^+. These values correspond well, as do the B_{50} values, with those for guinea pig brain in terms of both relative order and magnitude. The n values, at both 0 and 0.625 mM K^+ are in the range of 47-83 pmoles/mg protein. The n values, as given in Table 14-Preparation 1, are approximately one-half those determined for guinea pig brain, although the specific activities of the two enzymes are similar. The n values for Preparation 2 more closely approximate those for guinea pig brain.

The I_{50} concentrations are up to 100 times higher than those concentrations required for B_{50} (Table 15). This descrepancy could not be eliminated by pre-incubating the enzyme with glycoside, in normal medium, and then starting the reaction by the addition of K^+. It thus did not appear to be a problem of reaching equilibrium between the enzyme and glycoside.

When the effect of various K^+ concentrations on K_a and B_{50} values was studied (Figure 12 and Table 16) (see Appendix Tables 38-39) the results directly parallel those for guinea pig brains (Table 8). The K_a values decreased ($21.2 \rightarrow 0.56 \times 10^{-7}$ M^{-1}) with increasing K^+ concentration (0-20 mM) and the B_{50} values increased ($0.9 \rightarrow 18.0 \times 10^{-8}$ M) over the same K^+ concentration range.

To determine whether or not an equilibrium had been reached in the time period of the binding experiments and to ensure further that the binding was reversible, studies of association and dissociation of cardiac glycosides to and from rat brain ATPase were carried out. A constant b/u ratio (Figure 13) was obtained for convallatoxol-^{3}H at 1×10^{-8} M by five minutes and was maintained up to thirty minutes.

TABLE 15

A comparison of I_{50} and B_{50} values, Rat Brain*

This table compares selected data from Tables 13 and 14.

	I_{50}	B_{50}
	μM	μM
Digitoxin	2.1	.036
Cymarol	3.2	.028
Convallatoxol	1.7	.022
Digoxin	1.6	.084
Ouabain	3.2	.073

* [KCl], 0.625 mM

Figure 12. The effect of various K^+ concentrations on the high affinity binding of convallatoxol-^3H to rat brain Na^+K^+-ATPase.

Binding studies were carried out with rat brain Na^+K^+-ATPase (specific activity 24.0) and convallatoxol-^3H as described under Experimental Methods in the presence of various KCl concentrations (○ 0 K^+, ◘ 0.625 mM K^+, ✚ 2.5 mM K^+, ■ 5.0 mM K^+, ● 20 mM K^+) (see Appendix Table 39).

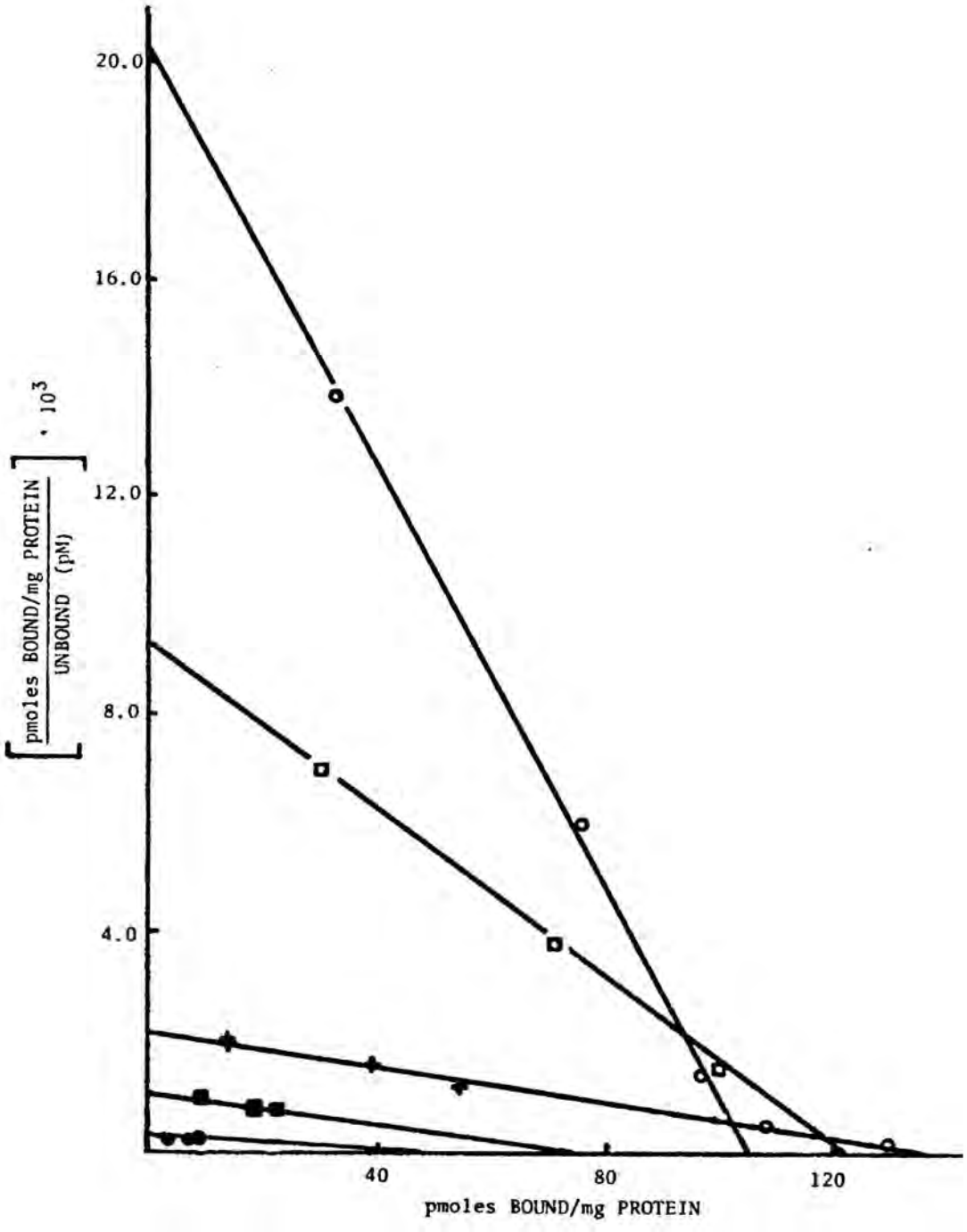

20.0
16.0
12.0
8.0
4.0
40
80
120
pmoles BOUND/mg PROTEIN
[pmoles BOUND/mg PROTEIN / UNBOUND (pM)] · 10³

TABLE 16

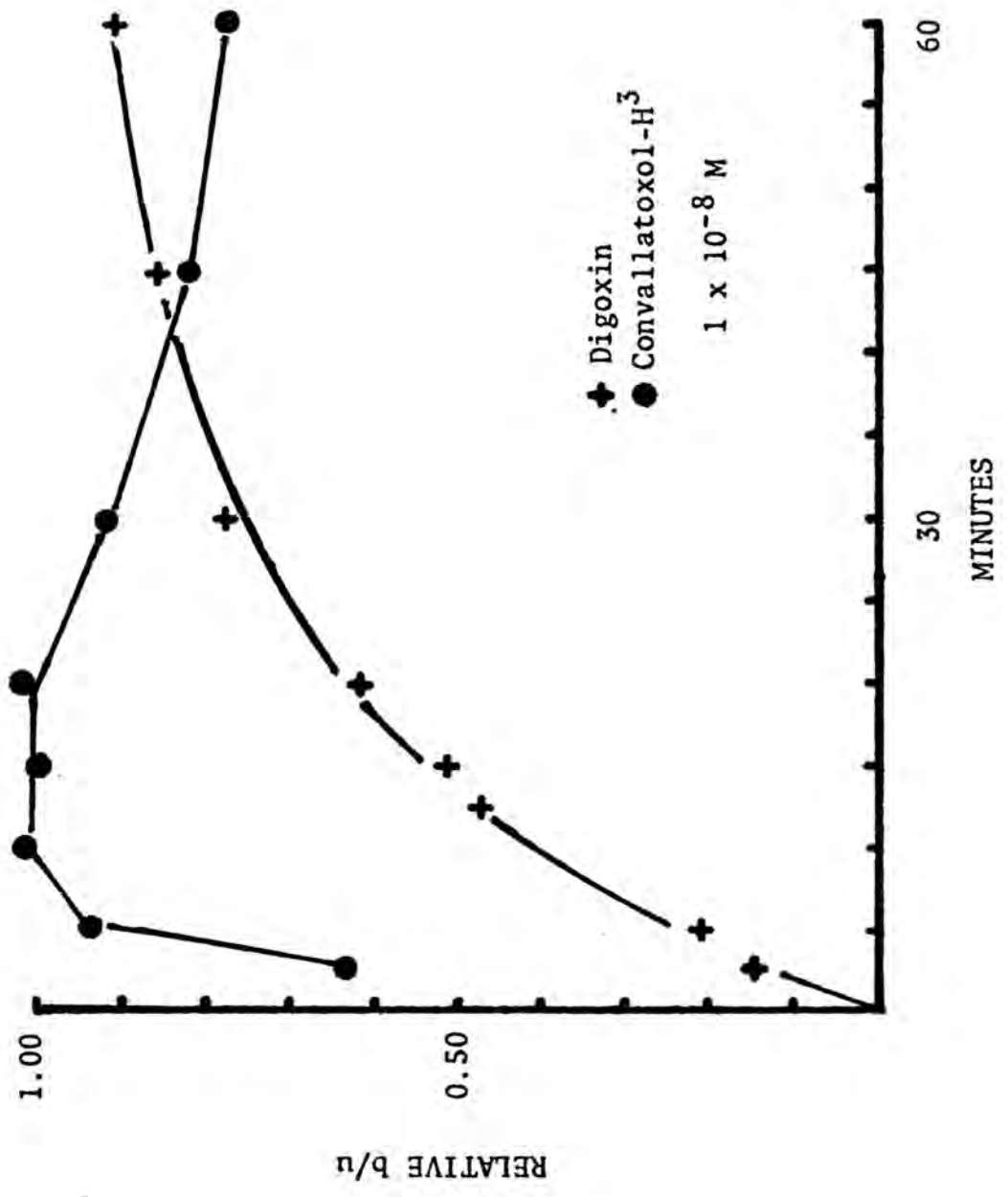
K_a and B_{50} values of rat brain Na^+K^+-ATPase as a function of K^+ concentration

The K_a and B_{50} values of rat brain Na^+K^+-ATPase (specific activity 24.0) with convallatoxol-^3H were calculated from Scatchard plots of data obtained at the stated concentrations (see Appendix Table 38).

$[K^+]$	$K_a, 10^{-7}$ M^{-1}	B_{50}
mM		µM
0.0	21.2	0.0094
0.625	7.67	0.0184
2.50	1.65	0.0664
5.00	1.43	0.0730
20.00	0.56	0.180

Figure 13. The rate of association of ^{3}H-glycoside to rat brain $Na^{+}K^{+}$-ATPase.

The association studies were carried out with rat brain $Na^{+}K^{+}$-ATPase (specific activity 29.4) and either convallatoxol-^{3}H (●) or digoxin-^{3}H (◘) as described under Experimental Methods for the specified times (see Appendix Tables 40-41).


1.00
0.50
RELATIVE b/u
30
60
MINUTES
Digoxin
Convallatoxol-H3
1 x 10-8 M

A relatively constant b/u ratio was obtained for digoxin-^{3}H at 1×10^{-8} M by thirty minutes and was maintained for another thirty minutes (Figure 13). When dissociation of glycoside-^{3}H from ATPase (Figure 14) in the presence of incubation medium with 1×10^{-5} M unlabeled glycoside was studied, a new b/u equilibrium value was reached in 30 minutes for both convallatoxol and digoxin with a major portion of the dissociation occurring by fifteen minutes. This dissociation represented 70 and 40%, respectively, of that glycoside-^{3}H which was bound in the presence of 1×10^{-8} M convallatoxol and digoxin. This is in comparison to all three guinea pig Na^+K^+-ATPases which maintained a constant b/u ratio for convallatoxol-^{3}H from 2-30 minutes.

C. Rat Heart Na^+K^+-ATPase.

Rat Heart Assay: As described for the rat brain, a linear production of P_i was observed (Figure 15) well past the time required for the assay and inhibition studies, as well as a linear increase in P_i production with increasing amounts of enzyme (Figure 16). A study of enzyme activity versus K^+ concentration (Figure 11) revealed that maximum Na^+K^+-ATPase activity occurred at 1.25 mM K^+ and that this activity was maintained up to 20 mM K^+.

Inhibition of Rat Heart Na^+K^+-ATPase by Various Cardiac Glycosides at 0.625 and 20 mM K^+: The concentration of glycoside which will yield 50% inhibition (Table 17) for 20 mM K^+ is 3-20 fold greater than the required glycoside concentration at 0.625 mM K^+. From these data it would appear that rat heart ATPase is least inhibited by convallatoxol and most inhibited by hellebrin at both K^+ concentrations. However, if these concentrations of glycoside (at 0.625 mM K^+) are compared with those required to obtain half-inhibition

Figure 14. The rate of dissociation of ^{3}H-glycoside from rat brain $Na^{+}K^{+}$-ATPase.

The dissociation studies were carried out for the specified times with rat brain $Na^{+}K^{+}$-ATPase (specific activity 29.4) and either convallatoxol-^{3}H (✤) or digoxin-^{3}H (✪) as described under Experimental Methods for the specified times (see Appendix Tables 40-41).

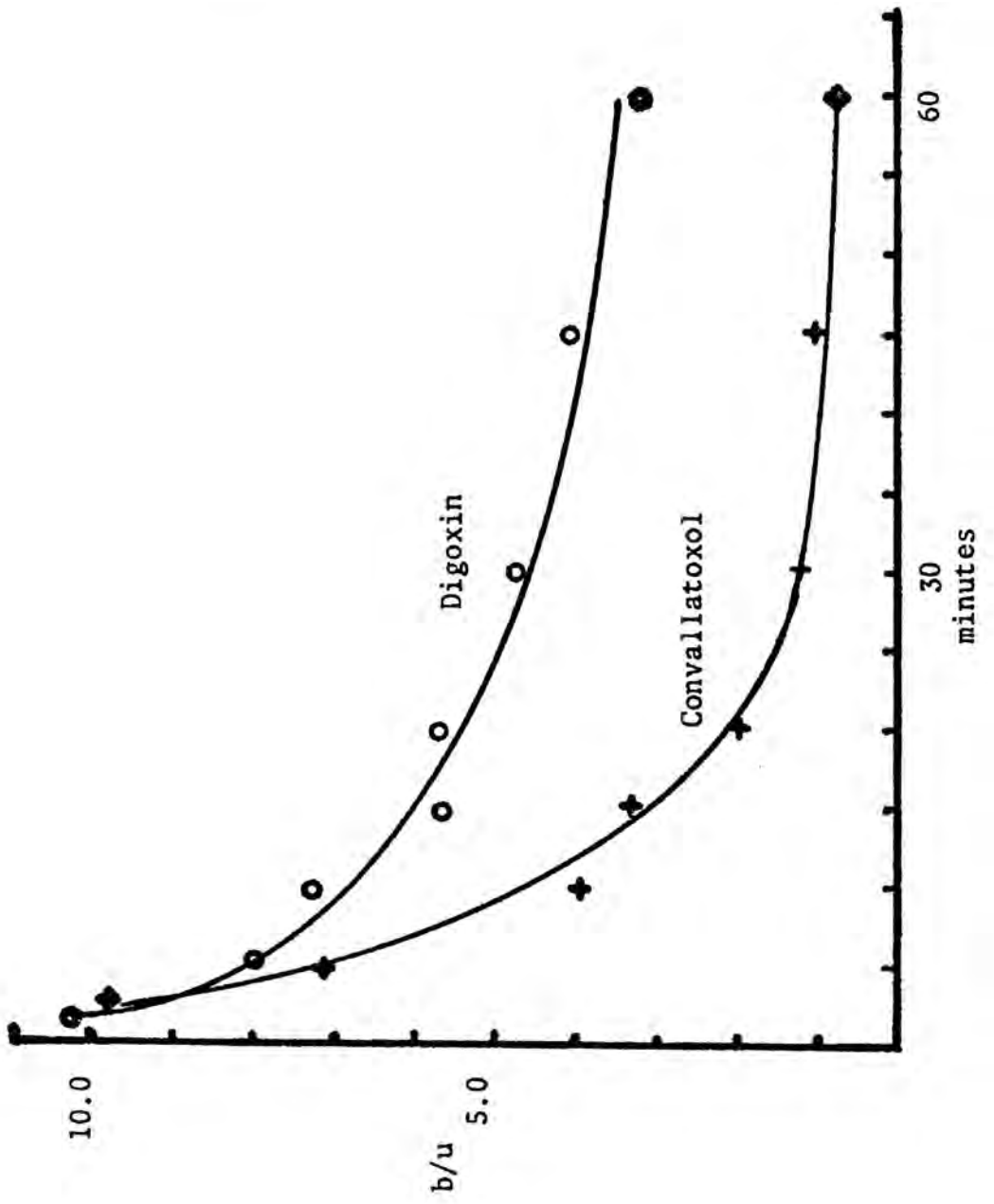

10.0
5.0
b/u
Digoxin
Convallatoxol
30
60
minutes

Figure 15. Hydrolysis of ATP by rat heart Na^+K^+-ATPase as a function of time.

The assay of rat heart Na^+K^+-ATPase (specific activity 9.2) was carried out as described under Experimental Methods (see Appendix Table 46).

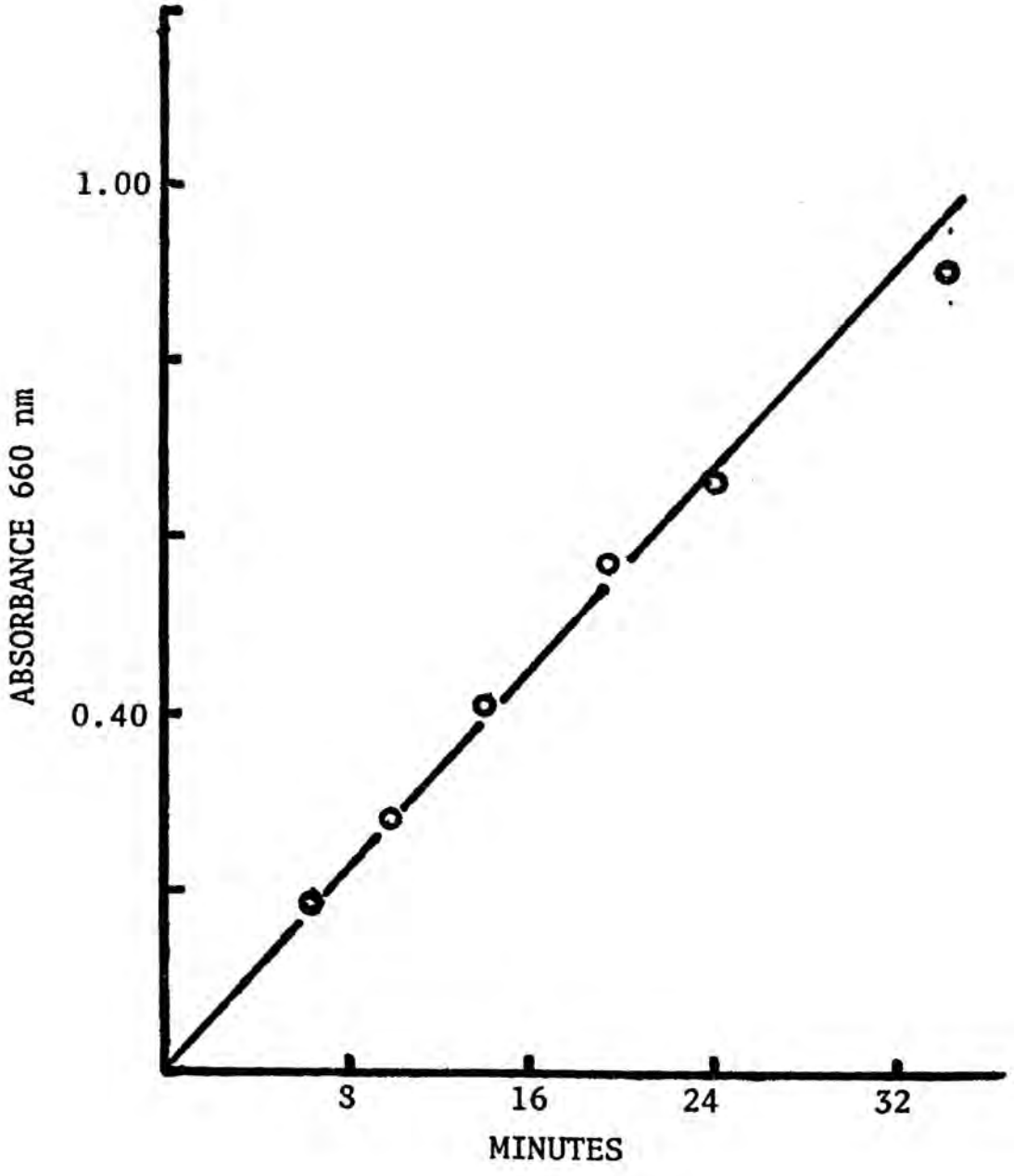

1.00
0.40
ABSORBANCE 660 nm
8
16
24
32
MINUTES

Figure 16. Rat heart Na^+K^+-ATPase activity as a function of enzyme concentration.

The assay of rat heart Na^+K^+-ATPase (specific activity 13.2; 2.0 mg protein/ml) was carried out as described under Experimental Methods with the specified volumes of enzyme solution (see Appendix Table 32).

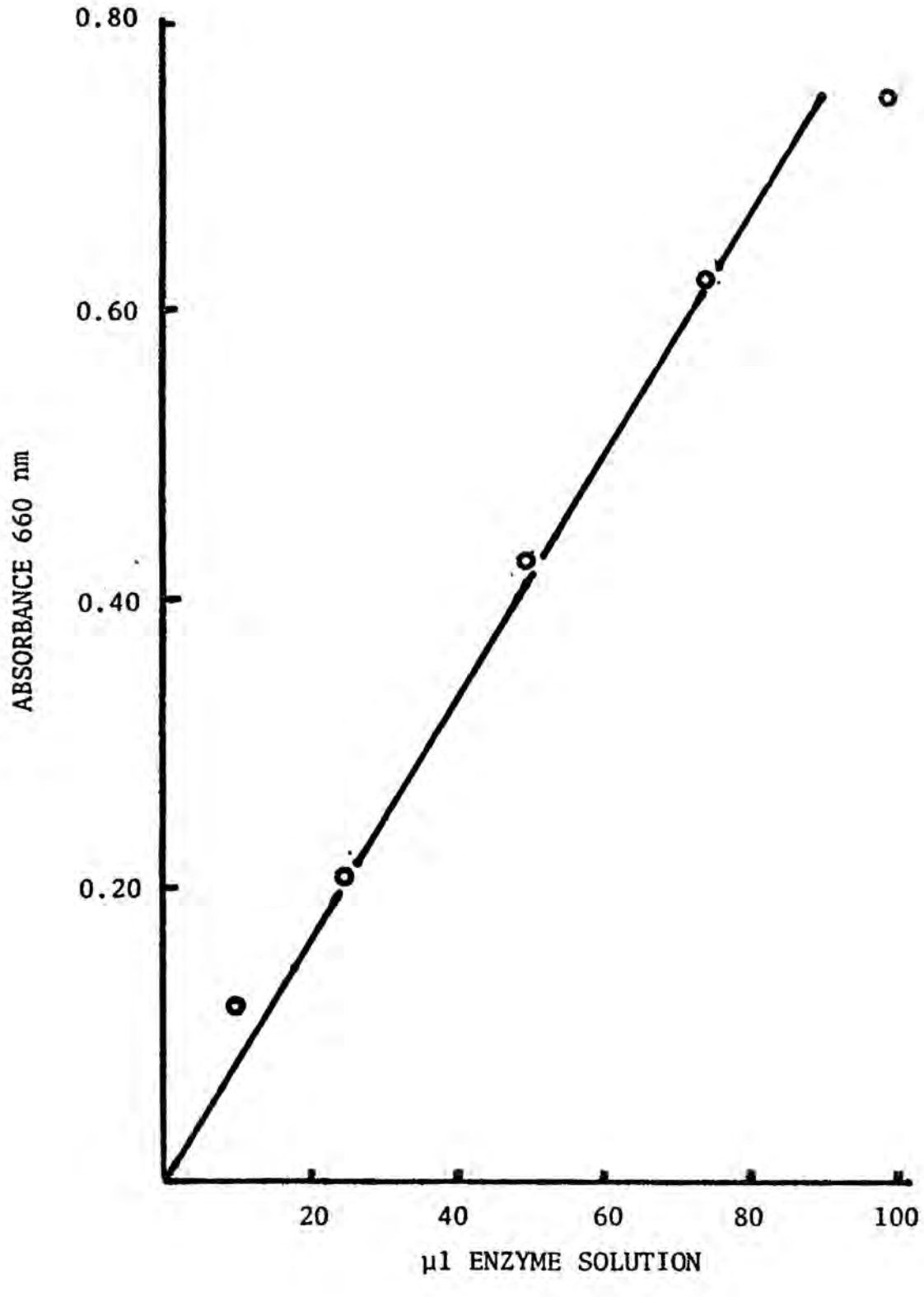

0.80
0.60
0.40
0.20
ABSORBANCE 660 nm
20
40
60
80
100
µl ENZYME SOLUTION

TABLE 18

A comparison between the binding of 1×10^{-8} M convallatoxol-^{3}H by rat heart and rat brain $Na^{+}K^{+}$-ATPase

The binding studies were carried out as described under Experimental Methods (Appendix Tables 48-52).

	Heart	Brain
b/u	0.027	21.0
mg/Protein	0.165	0.050
S.A. µmoles P_i/hr/mgProtein	9.0	38.3

DISCUSSION

In order to better understand the interrelationships between the important group of drugs, the cardiac glycosides, and Na^+K^+-ATPase, there are several questions of a quantitative nature which must be answered. They are: (1) Can the inhibition of guinea pig and rat Na^+K^+-ATPases by cardiac glycosides and the binding of glycosides be related quantitatively? In other words, under identical conditions does 50% inhibition occur when a recognizable binding site is 50% saturated? (2) Are there fundamental differences in the sensitivities of Na^+K^+-ATPases from different organs and species to inhibition by the glycosides and do these different Na^+K^+-ATPases bind glycosides with different affinities? (3) Do glycosides with differing physical, chemical and pharmacologic properties bind to Na^+K^+-ATPase with different affinities? (4) Can the binding affinities be related to pharmacologic potencies or onsets and durations of action? The results described in this dissertation bear on this series of questions.

Discussion of Guinea Pig ATPases: The Na^+K^+-ATPase activity for guinea pig heart, kidney and brain was dependent upon ligand concentrations, time and temperature. Under the conditions used, the enzymes displayed linear production of P_i over the time period employed in the inhibition and binding studies. With all other ligands at constant concentrations, changes in K^+ concentration produced changes in enzymatic activity of all three enzymes. It was

demonstrated that at 0.625 mM K^+ there was sufficient activity in each enzyme to carry out the inhibition studies. This was important in that the inhibition studies were to be compared to binding studies at the same K^+ concentrations and the normal K^+ concentrations prevented binding. It should be noted that the activity for heart ATPase at this low K^+ concentration was only 45% of its maximal activity. As a result of the decreased activity of the heart ATPase at 0.625 mM K^+ there is a probable increased error in the estimation of the I_{50}. This could explain some of the variability seen in comparisons of the I_{50} values and B_{50} values determined at this K^+ concentration (Table 10).

The effect of K^+ on ATPase inhibition by cardiac glycosides is dramatic. I_{50} values are 30-200 fold lower for brain, 20-80 fold lower for kidney, and 25-250 fold lower for heart Na^+K^+-ATPase when the K^+ concentration is decreased from 20 mM to 0.625 mM (Table 5). Repke (8) showed some decrease in the I_{50} for ouabain with guinea pig heart Na^+K^+-ATPase when K^+ concentration was reduced from 25 mM to 2.5 mM. The observations herein are also consistent with those made by Akera (110) where the I_{50} value for ouabain was lower when 15 mM K^+ was added at the end of a preincubation period rather than with the glycoside. The relative order of I_{50} values among the various cardiac glycosides was essentially the same for each Na^+K^+-ATPase at 0.625 mM K^+. Kidney ATPase had I_{50} values which were 5 to 9 fold larger than the values for the same glycoside inhibition of brain or heart.

Binding experimental data can be treated in numerous ways (see earlier discussion). The Scatchard method was chosen to plot the experimental data because it best deals with data containing multiple

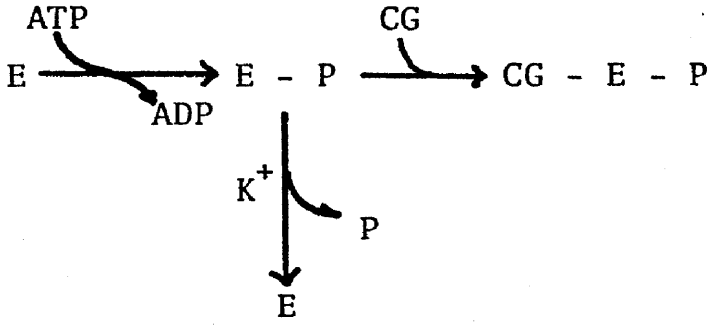
sets of binding sites. The Scatchard plot places nearly equal emphasis on the binding parameters of all sets of sites. When using the Scatchard plot the experimental data of the binding of various glycosides to Na^+K^+-ATPase can be discussed in terms of three parameters -- the affinity constant, K_a; the concentration at which the high affinity site is half-saturated, B_{50}; and the maximum number of pmoles CG bound/mg protein, n (see earlier discussion). Since the Scatchard method is dependent upon the existence of an equilibrium, it is important to note that maximum binding to the guinea pig enzymes (heart, brain and kidney) is reached by 200 seconds (102), well within the time period of the binding experiments.

It was demonstrated that the binding of cardiac glycosides to Na^+K^+-ATPase of all three guinea pig tissues was dependent upon well established requirements for ATP, Mg^{2+} and Na^+ (1,7,10-13). It was shown that the slope of the high affinity portion of the Scatchard plot was greatly reduced when any one of the above ligands was omitted. Furthermore, the inclusion of K^+ had the same effect. This latter was to be expected, since it has been clearly demonstrated that K^+ enhances the hydrolysis of the phosphorylated enzyme, the form of the enzyme to which glycosides normally bind (7,9,35,83,84,98).

In order to compare inhibition and binding data under equivalent conditions, it was necessary to do the binding studies in the presence of the same K^+ concentration as used in the inhibition studies. A concentration of K^+ which allowed for both enzymatic activity and significant high affinity binding was arrived at, 0.625 mM K^+.

To delineate further the effect of K^+ on the binding of cardiac glycosides by Na^+K^+-ATPase from the three guinea pig tissues, two

studies were carried out. The first demonstrated a marked decline in the b/u ratio at a fixed glycoside concentration (1×10^{-8} M) when the K^+ levels were increased from 0 to 20 mM (Appendix Table 14). It was noted that there were still relatively high b/u ratios at 0.625 mM K^+, indicating that it should be possible to do binding studies at this K^+ concentration. The second study yielded a family of curves obtained from Scatchard plots of binding studies done in the absence of K^+ and in the presence of various K^+ concentrations (Figure 8). It was observed from these curves that increasing K^+ concentration reduced K_a values and in turn produced an increase in the B_{50} values; i.e. when the 'apparent affinity' was reduced, a higher concentration of glycoside was required to half-saturate the high affinity site. This most probably is a result of the stimulatory effect of K^+ on the dephosphorylation reaction which leads to the breakdown of the active binding species of the enzyme. In the absence of K^+ all the enzyme species presumably exist in an equilibrium situation; in the presence of excess ATP and the absence of K^+, the enzyme should exist primarily



in two forms, E - P and CG - E - P, which are in equilibrium with each other. This is consistent with the fact that at a fixed glycoside concentration (1×10^{-8} M) the b/u ratio does not change over an extended time interval (2-30 min.) (Appendix Tables 42-45). When K^+ is added, the enzyme is partially dephosphorylated; this may lead to

the existence of a significant amount of the enzyme in the free form. Thus, in the presence of K^+ there exist three enzyme forms, E, E - P, and CG - E - P; there also exists a new set of conditions where the phosphorylated form of the enzyme no longer exists in a true equilibrium condition but in a quasi-equilibrium or steady state, since the phosphorylated form is being continually hydrolyzed and rephosphorylated. The fact that there is good agreement between the equilibrium binding data (B_{50}) and the kinetic inhibition data (I_{50}) at 0.625 mM K^+ indicates that a steady state does exist (Table 11). As the concentration of K^+ is increased a corresponding decrease in the steady state level of the phosphorylated enzyme species occurs, accounting for the diminished n values of the Scatchard plot (Figure 8). Under the above conditions it is expected that n should decrease in the presence of K^+, but that the affinity for the remaining sites should remain the same as in the absence of K^+. The 'apparent K_a' decreases in the presence of K^+ (Figure 8). This may reflect a true decrease in the affinity of the enzyme for the glycoside but more probably results from the n value being influenced by the competition between the glycoside and K^+ for the phosphorylated enzyme, E - P. As the concentration of glycoside is increased at constant K^+ concentration the concentration of the CG - E - P form of the enzyme will increase leading to a concomitant decrease in E - P and E, establishing a new steady state condition. The greater the concentration of glycoside, in the presence of K^+, the greater will be the amount of enzyme in the CG - E - P plus E - P forms as compared to the E form. Thus, the higher the glycoside concentration, the closer the value of n in the presence of K^+ approaches the value of n in the absence of K^+.

Therefore the Scatchard plot of glycoside binding to Na^+K^+-ATPase in the presence of K^+ will reflect a slope change (K_a change) due to the variable effect of glycoside/K^+ competition in the steady state on the value of n. In other words, the high K^+ concentration reduces the apparent affinity of the ATPase for the glycosides, by diminishing the 'steady state' concentration of the enzyme form to which the tritiated cardiac glycoside binds. Therefore, in the presence of K^+ the apparent K_a is a measure of more than the affinity constant of the cardiac glycoside binding reaction.

With all of this in mind, it is still possible to make comparisons of the interactions of the various glycosides with a specific Na^+K^+-ATPase and also relative comparisons among enzyme preparations in terms of the interactions with the glycosides employing the binding parameters K_a, n and B_{50}. The affinity constants, K_a, for the high affinity sites of guinea pig kidney and heart Na^+K^+-ATPase are quite similar in the absence of K^+ and at 0.625 mM K^+. This is true for all the glycosides. The K_a for guinea pig brain Na^+K^+-ATPase is somewhat higher, showing that the enzyme in this organ binds the glycoside more strongly.

The affinity of Na^+K^+-ATPase for ouabain and digoxin is consistently lower for all three tissues than the affinity for convallatoxol, cymarol and digitoxin in the absence of K^+. In the presence of 0.625 mM K^+ the difference between these two groups is less exaggerated, with heart ATPase displaying the least difference and brain the greatest. This suggests that the more active enzyme is more sensitive to small changes in K^+ concentration.

The number of binding sites per mg of enzyme protein roughly

correlates with the Na^+K^+-ATPase specific activities, being highest for brain and lowest for heart (Table 9). This suggests that the number of binding sites per molecule of enzyme may be similar for all three preparations but more precise studies with more highly purified enzyme preparations are needed to prove this point. As the specific activity of Na^+K^+-ATPase decreases with aging for a given preparation the n values also decrease, but at a slower rate (Table 10). It is possible that the binding capacity of the enzyme is not dependent upon the hydrolyzing capacity. In other words the ability to break down E - P does not appear to be required in order for binding to occur. Although the enzyme system may have to remain intact, the phospholipid subunit may not have to be entirely functional in order for the glycopolypeptide subunit to bind glycoside.

Harris et al. (88,111) have demonstrated ouabain binding by a membrane fraction from beef brain that is low in ATPase activity. They suggested that this represented an inactive form of the enzyme resulting from a loss of phospholipid without concomitant loss of binding capacity. Taniguchi and Iida (112) have similarly suggested that phospholipid is necessary for ATPase activity but not for ouabain binding.

The B_{50} values in the absence of K^+ for all three tissues fall in the 3-21 x 10^{-8} M range. With the exception of four values, the values all fall in the 3-10 x 10^{-8} M range. The B_{50} values for ouabain and digoxin binding to kidney and heart ATPases are slightly higher than the values for the other glycosides. Upon the inclusion of 0.625 mM K^+, the range of B_{50} values broadens to 3-61 x 10^{-8} M for all three organs. With the exception of the kidney B_{50} values,

which appear quite sensitive to K^+ change, the range is 3-16 x 10^{-8} M. The B_{50} values for kidney Na^+K^+-ATPase double or triple, while those for brain and heart are not significantly changed. The kidney is the only organ of the three that maintains the majority of its activity at this low K^+ concentration (Figure 6). The greater effect of 0.625 mM K^+ on the binding, relative to that at 0 K^+, is consistent with the fact that 0.625 mM K^+ supports almost maximal activity of the kidney enzyme and not of the other two. This concentration causes a greater reduction in the level of the phosphorylated form of the kidney enzyme and a resulting larger decrease in K_a and increase in B_{50}.

The concentrations at which specific ouabain binding to guinea pig kidney enzyme occurs are similar to those reported by Tobin and Sen (92) and Erdmann and Schoner (95). The apparent K_m value reported by the former group, 1.8 x 10^{-7} M, compares with the B_{50} value found in this work of 2.1 x 10^{-7} M, and the value, K_d = 1.6 x 10^{-7} M of the latter group, compares with the K_a of 5.4 x 10^6 M^{-1} (K_d = 1.8 x 10^{-7} M) found in this study. The K_a and n values for ouabain binding to guinea pig brain Na^+K^+-ATPase (Table 9) are in reasonably good agreement with those reported by Taniguchi and Iida (93) for ox brain Na^+K^+-ATPase, K_a = 5.5 x 10^6 M^{-1}, n = 119 pmoles/mg protein. The K_a values found in the present study for kidney and heart ATPase binding of ouabain are, however, closer to the values of Taniguchi and Iida (93). These comparisons are made to point out the similarities between some species in terms of glycoside/Na^+K^+-ATPase interactions.

When a comparison of individual I_{50} values with the corresponding B_{50} values is made for each of the preparations, there is reasonably

good agreement (Table 11). When the possible sources of error are taken into account, it is probable that the majority of values are not significantly different in an I_{50} to B_{50} comparison. It is interesting to note that, the heart I_{50}/B_{50} ratios for a particular glycoside are not significantly different from those for the brain although the ratios for kidney do differ somewhat. The I_{50}/B_{50} values among the five glycosides do not differ significantly with the enzyme from brain or heart. The kidney preparation on the other hand appears to have a somewhat lower affinity for the glycosides as a group and in particular for digoxin and ouabain. Even so, the agreement between tissue preparations is fairly good considering that each enzyme was not highly purified, but is simply a suspended membrane preparation.

These data would suggest that there is a one to one relationship of the binding to the inhibition of guinea pig preparations. This could be interpreted to mean that the high affinity binding site is the inhibitory site of the enzyme. Various studies have led to the conclusion that Na^+K^+-ATPase contains 1, 2 or even more sites, dependent upon the enzyme source. These values have been surmised by comparing ouabain binding to specific activity (75,87) and to the number of phosphorylations (77); Scatchard plots of ouabain binding to ox brain ATPase have shown two binding sites, one with a K_d of 0.18×10^{-6} and the other K_d of 20×10^{-6} (81,93), while ox kidney on the other hand appears to have one high affinity receptor, K_d, 0.47×10^{-8} M (95). Erdmann and Schoner (95) reported that guinea pig kidney has four ouabain sites per phosphorylated intermediate, although they point out that the ouabain receptor and the ATP

Inorganic phosphate, in the presence of Mg^{2+}, will promote binding of cardiac glycosides to much the same extent as does Mg^{2+}, Na^+ and ATP (Table 12). In our limited studies of this phenomenon we have found the binding to be similar under these quite different procedures; the high affinity binding parameters are similar. This binding is inhibited not only by K^+ but also by Na^+ at a concentration that is required for the ATP promoted binding. These results are consistent with those of other investigators (65,78,83,86,88,92,95, 103). All these results strongly suggest that a phosphorylated intermediate of the enzyme is formed in the presence of P_i and that this intermediate is similar, if not identical, to the glycoside binding intermediate formed with ATP. Tobin and Sen (92) reported ouabain-^3H binding to guinea pig kidney Na^+K^+-ATPase in the presence of Mg^{2+} and P_i which was saturated at 1×10^{-5} M and was inhibited by Na^+ and K^+. Equilibrium was reached in three minutes at 37°C. Erdmann and Schoner (95) have reported a K_d of 1.62×10^{-7} M for guinea pig kidney Na^+K^+-ATPase at 37°C.

Discussion of Rat ATPases: As was true with the guinea pig Na^+K^+-ATPases, rat brain and heart Na^+K^+-ATPase activity is dependent on time, temperature and ligand concentration. The enzymes displayed linear P_i production over the times employed in the inhibition and binding studies. Keeping all other ligands at constant concentration, changes could be observed in enzymatic activity of both rat heart and brain ATPase when the K^+ concentration was varied. The optimum K^+ concentration for rat heart ATPase was 2.5 mM and for brain 5.0 mM. It was shown that sufficient activity remained at 0.625 mM K^+ for inhibition studies to be carried out. As with the guinea pig enzymes,

activity at this K^+ concentration was important because of the comparison needed between inhibition and binding studies at the same K^+ concentration.

Lowering the K^+ concentration from 2.5 to 0.625 mM has little effect on the I_{50} values for the enzyme from rat brain. The changes were negligible in comparison to the differences observed with the guinea pig enzymes. It appears that rat brain Na^+K^+-ATPase is insensitive to K^+ concentration changes in terms of its inhibition by cardiac glycosides. The I_{50} values for rat brain are in the range of 1-5 x 10^{-6} M; these values are consistent with the value of 1.6 x 10^{-6} M (in the presence of 15 mM KCl) reported for ouabain by Tobin and Brody (94). Shirachi, Allard and Trevor (104) reported 42% inhibition by 1 x 10^{-6} M ouabain in the presence of 20 mM K^+ and 48% in the presence of 10 mM K^+.

A change in K^+ concentration from 20 to 0.625 mM caused a decrease in the I_{50} values for rat heart Na^+K^+-ATPase. The I_{50} range changed from 32-500 μM to 3-190 μM when the K^+ concentration was lowered from 20 to 0.625 mM (Table 17). The largest difference was approximately 20 fold, for digoxin, and the smallest was approximately five fold. Although these differences for rat heart are not as large as those observed for the guinea pig preparations, they are appreciable. The lack of a K^+ effect distinguishes rat brain from rat heart Na^+K^+-ATPase and from all of the guinea pig enzymes studied. Both rat heart and brain Na^+K^+-ATPase require more glycoside to attain I_{50} than do the guinea pig preparations with brain requiring 10 to 100 fold more glycoside and heart 300-1000 fold more glycoside.

Does the binding of glycosides to the rat Na^+K^+-ATPase reflect

these I_{50} values? The affinity constants for rat brain are very similar in the absence of K^+ or in the presence of 0.625 mM K^+. They range from 1.4 to 7.6 x 10^7 M^{-1} in the presence of 0.625 mM K^+ which shows only a very small change from the values obtained in the absence of K^+. These K_a values for rat brain (Table 14) are essentially the same as those for guinea pig brain (Table 9). Of the other binding parameters, B_{50} values are also similar for rat brain and guinea pig brain enzyme. It is concluded that the high affinity sites of the two enzymes are similar with respect to their affinity for glycosides. The n values for rat brain are significantly lower than those for guinea pig brain, suggesting rat brain binds less glycoside per mg protein. This is in contrast to the fact that the specific activities of fresh preparations of each enzyme are approximately the same.

A large discrepancy between I_{50} and B_{50} values is observed with the enzyme from rat brain. It requires 100 fold more glycoside to half-inhibit the enzyme than to half-saturate the high affinity site (Table 15). This is in marked contrast to the guinea pig enzymes, where under identical conditions the I_{50} and B_{50} values were comparable. Preincubation of the rat brain enzyme with glycoside did not lower the I_{50} value. Thus it appears that failure for equilibrium to be reached does not explain this discrepancy. A further indication that the data cannot be explained by a lack of equilibrium is from the fact that the two glycosides which would be expected to take longest to reach equilibrium (Figure 13, Table 3), digoxin and ouabain[3], show only 20 and 40 fold differences while the other three show approximately 100 fold differences. It appears that a second site

of lower affinity (the inhibitory site) may be involved which has about the same affinity for all the glycosides. Two ouabain binding sites have been reported for ox brain by Taniguchi and Iida (93)--1) K_d = 0.18 μM, n = 119 pmoles/mg protein and 2) K_d = 20 μM, n = 104 pmoles/mg protein. They found that ouabain binding to higher affinity site caused a marked reduction in Na^+K^+-ATPase activity (0.18 μM ouabain). Their experiments were done in the presence of 14 mM KCl and required 2 hours incubation before the binding reached a plateau. The number of lower affinity binding sites was reduced in half by carrying out the binding experiments in the absence of K^+. Although these observations are interesting to note, especially the existence of two distinguishable ouabain binding sites, it is difficult to correlate them with the results noted here for rat brain enzyme. To answer the question posed earlier, it is not clear that the explanation for low ouabain sensitivity to glycosides by rat brain enzyme can be explained in terms of its high affinity binding. The rat brain therefore presents a real enigma. Rat heart Na^+K^+-ATPase, on the other hand, presents a clearcut case. The rat heart simply does not possess a Na^+K^+-ATPase which can bind the glycosides at high affinity. Under various conditions, with and without K^+ and over an extended time interval, no specific binding of cardiac glycosides could be demonstrated to rat heart Na^+K^+-ATPase. This would correspond well with the fact that at least 3 μM digoxin and up to 190 μM convallatoxol were required to half-inhibit the enzyme. This concentration range is well out of the high affinity binding range observed for rat brain and all the guinea pig enzymes. Taniguchi and Iida (93) postulated that there was "cooperativity" between the two ouabain binding sites

in ox brain. It is possible that an analogous situation exists in the rat brain, such that the inhibitory site (lower affinity site) may not bind without a binding to the higher affinity site occurring first. Thus, if the rat heart were considered in this light, it could be speculated that the high affinity site of the rat heart Na^+K^+-ATPase is functionally missing. Due to the absence of this site, the lower affinity site cannot interact cooperatively; therefore, the I_{50} concentrations are greater than those observed for rat brain Na^+K^+-ATPase.

CONCLUSIONS

The results herein seem to be consistent with the generally accepted model for the interactions between Na^+K^+-ATPase, ATP and cardiac glycosides in a suitable ionic environment. Omission of ATP, Mg^{2+} or Na^+ eliminates the high affinity binding. Inclusion of Mg^{2+} and P_i in the absence of ATP promotes binding. It appears that a phosphorylated form of the enzyme, produced by its interaction with ATP in the presence of Mg^{2+} and Na^+ or P_i in the presence of Mg^{2+}, is the binding species. The binding was shown to be freely reversible, in contrast to some of the results reported by others, who used ATPase preparations from other species. Tobin and Sen (92) also found the binding of ouabain to guinea pig kidney Na^+K^+-ATPase to be reversible.

It would appear that the inhibitory effect of K^+ on binding results from its role in promoting the hydrolysis of the phosphorylated form of the enzyme. Similar observations have been made by Baker and Willis (113) on the binding of ouabain by HeLa cells. With increasing K^+ concentrations there is a decrease in the steady state level of phosphorylated enzyme which is, in turn, in equilibrium with the form bound to the glycoside. This leads to an apparent reduction of the values of n and K_a in the presence of 0.625 mM K^+ and the complete disappearance of the high affinity binding at 20 mM K^+. These results support other observations herein that the binding of Na^+K^+-ATPase to cardiac glycosides is freely reversible. If reversibility were not the case, the binding would represent a 'trap' for the phosphorylated

enzyme and eventually all enzymatic activity could be abolished even at low glycoside concentrations (1×10^{-7} M). It is interesting to note that several groups have suggested that the extent of binding is not affected even in the presence of large K^+ concentrations if enough time is allowed for an equilibrium to be established.

By using the Scatchard method of analyzing the data, with each glycoside and each guinea pig Na^+K^+-ATPase and rat brain preparation, it is possible to show a high affinity binding site which requires ATP, Mg^{2+} and Na^+ and which is eliminated by high concentrations of K^+. By selecting a low concentration of K^+, we have found conditions under which both binding and Na^+K^+-ATPase inhibition can be studied. Under these conditions, 50% inhibition of the enzymic activity and 50% saturation of the high affinity binding site both occur at similar glycoside concentrations for all glycosides and all guinea pig enzyme preparations. This was not true for rat brain Na^+K^+-ATPase where the I_{50} values were a hundred fold greater than the B_{50} values. No comparison can be made for rat heart since it displays no high affinity binding. This virtually established for the guinea pig enzymes that the binding of the glycosides is by the Na^+K^+-ATPase in the crude preparations and that the binding site is the inhibitory site. No contaminating substance in any of the preparations shows appreciable high affinity binding. It follows that the high uptake *in vivo* of glycosides by heart and kidney (36-38) is most probably a result of binding to the inhibitory site of Na^+K^+-ATPases. It thus appears warranted to consider this site on the enzyme as the receptor for cardiac glycosides. The uptake of glycosides by brain *in vivo* is quite low (17), presumably as a result of the blood-brain barrier,

even though the enzyme from this source still has the capacity to bind glycosides.

The rat brain Na^+K^+-ATPase may have a requirement for more than one molecule of cardiac glycoside for inhibition. It might possess two binding sites for the glycoside, similar to the two sites reported for ox brain, one with an affinity 100 fold greater than the other. The lack of any apparent high affinity binding by rat heart Na^+K^+-ATPase supports the role of Na^+K^+-ATPase as the receptor for and mediator of cardiac glycoside action, since the rat is a glycoside insensitive species.

Comparisons among the five glycosides studied reveal no correlations between any binding parameter and any parameter of pharmacologic activity. In general the compounds fall into two groups -- digitoxin, cymarol and convallatoxol, which show relatively higher affinities than digoxin and ouabain. Ouabain, convallatoxol and cymarol are similar pharmacologically in that each has a very high potency and a rapid onset and short duration of action in vivo, whereas digitoxin is at the opposite extreme with respect to each of these properties. Digoxin is intermediate. No detailed correlation between chemical structure and binding affinity is made with this small number of compounds, although it should be noted that the two compounds with substituents on ring C, ouabain, with an 11α-hydroxyl and digoxin, with a 12β-hydroxyl, are least tightly bound. Additional studies to determine whether this indicates that ring C is of particular importance in the binding of the steroid to the enzyme surface would be interesting.

FOOTNOTES

1 Lefler, C. F. and B. Baggett, unpublished results.

2 One preparation of rat heart ATPase did show a small amount of high affinity binding, with a $K_a = 6.7 \times 10^7 M^{-1}$ and an n value of 4 for convallatoxol (see Appendix Table 51). The hearts appeared engorged with blood; therefore, the observed high affinity binding may have been due to a small amount of RBC ATPase which remained during the ATPase isolation process. A $K_a = 0.7 \times 10^7 M^{-1}$ has been reported for human RBC Na^+K^+-ATPase (100). An alternate explanation may be that there is a very small but finite amount of glycoside sensitive Na^+K^+-ATPase in rat hearts, while the bulk of the ATPase is glycoside insensitive.

3 From Figure 13 it can be seen that the rate of association of digoxin to Na^+K^+-ATPase is slower than that of convallatoxol. Data given in Table 3 for beef brain show that the rate of association of ouabain is half that for either convallatoxin, cymarin, or digitoxin. These data point out that digoxin and ouabain associate to ATPase at a slower rate than do the other glycosides mentioned. Since ATPase activity is measured over a ten minute period of time (starting at time = 0), a glycoside that binds slowly would exert a smaller inhibitory effect on ATPase than one that binds rapidly, even though both are equally bound at equilibrium. Therefore, a glycoside with a low rate of association would show a higher I_{50} relative to B_{50} than one with a high rate of association.

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APPENDIX

TABLE 1 . Guinea Pig ATPase Activity as a Function of Enzyme Concentration

µl Enzyme Solution	Heart[a]		Brain[b]	
	A_{660}	Activity	A_{660}	Activity
10	.120	5.1	.293	11.5
	.138	5.9	.342	13.4
	.126	5.4	.379	14.9
25	.246	10.5	-	-
	.232	9.9	.63	24.8
	.232	9.9	.64	25.1
50	.42	18.0	.95	37.3
	.44	19.0	.95	37.3
	.44	19.0	.95	37.3
100	.76	32.5	1.30	51.1
	.78	33.3	1.28	50.3
	.76	32.5	1.26	49.5
125	.89	38.0	1.40	55.0
	.94	40.2	1.40	55.0
	.91	38.9	1.40	55.0
150	1.00	42.7	1.40	55.0
	1.00	42.7	1.40	55.0
	1.00	42.7	1.46	57.4

[a] 1.25 A_{660} units/µmole P_i; Na^+K^+-ATPase S.A. - 11.1; .146 mg Pro/50 µl.

[b] 1.34 A_{660} units/µmole P_i; Na^+K^+-ATPase S.A. - 37.2; .148 mg Pro/50 µl.

TABLE 2 . Guinea Pig Heart ATPase Activity as a Function of KCl concentration

KCl mM	A_{660}	Total Activity[a]	A_{660}	Mg^{2+} Activity[b]
0.155	.264	11.3	.223	9.5
	.268	11.4	.233	10.0
	.256	10.9	.225	9.6
	.258	11.0	.236	10.1
0.310	.290	12.4	.220	9.4
	.247	10.6	.228	9.7
	-	-	.243	10.4
	-	-	.229	9.8
0.625	.347	14.8	.220	9.4
	.330	13.7	.213	9.1
	.325	13.9	.207	8.6
	.329	14.1	.212	9.1
1.25	.44	19.0	.223	9.5
	.41	17.5	.234	10.0
	.41	17.5	.215	9.2
	.40	17.3	.225	9.6
2.50	.51	21.7	.222	9.5
	.47	20.0	.216	9.2
	.48	20.4	.225	9.6
	-	-	.226	9.7
5.00	.43	18.2	.189	8.1
	.44	18.7	.185	7.9
	.42	18.0	.189	8.1
	-	-	.187	8.0
10.00	.41	17.4	.197	8.4
	.41	17.4	.197	8.4
	.40	17.2	.192	8.2
	.40	17.2	.199	8.5
20.00	.397	17.0	.207	8.8
	.376	16.1	.197	8.4
	.382	16.3	.202	8.6
	.368	15.7	.206	8.8

[a] 1.25 A_{660} units/μmole P_i; Na^+K^+-ATPase S.A. - 11.1

[b] inhibited with 5×10^{-4} M Ouabain.

TABLE 3 . Guinea Pig Brain ATPase Activity as a Function of KCl Concentration

KCl mM	A_{660}	Total Activity[a]	A_{660}	Mg^{2+} Activity[b]
Study 1				
0.155	.38	14.9	.170	6.7
	.37	14.6	.210	8.3
	.41	16.0		
	.42	16.5		
0.310	.51	20.1	.150	5.9
	.52	20.0	.149	5.9
	.49	19.3		
	.50	19.7		
0.625	1.01	39.7	.122	4.8
	1.01	39.7	.116	4.6
	1.09	42.9		
	1.15	45.2		
1.25	.84	33.2	.115	4.5
	.86	33.8	.122	4.8
	.83	32.6		
	.86	33.8		
2.50	.83	32.6	.137	5.4
	.82	32.4	.147	5.8
	.77	30.3		
	.81	31.8		
5.0	.94	37.2	.139	5.5
	.94	37.2	.157	6.2
	.93	36.6		
	.92	36.6		
10.0	1.01	39.7	.158	6.2
	1.02	40.1	.139	5.5
	1.00	39.3		
	1.02	40.1		
20.0	1.00	39.0	.130	5.1
	1.00	39.0	.145	5.7
	1.00	39.0		
	1.00	39.0		

[a] 1.34 A_{660} units/μmole P_i; Na^+K^+-ATPase S.A. - 37.2.

[b] inhibited with 5×10^{-4} M Ouabain.

TABLE 3 (continued)

KCl mM	A_{660}	Total Activity[a]	A_{660}	Mg^{2+} Activity[b]
Study 2				
0.155	.245	9.4	.110	4.2
	.229	8.7	.105	4.0
	.302	11.5	.110	4.2
0.310	.314	12.0	.105	4.0
	.311	11.9	.102	3.9
	-	-	.102	3.9
0.625	.48	18.1	.099	3.8
	.50	19.1	.110	4.2
	-	-	.099	3.8
1.25	.60	22.9	.105	4.0
	.62	23.7	.098	3.7
	.61	23.3	.101	3.8
2.5	.67	25.6	.101	3.8
	.69	26.3	.112	4.3
	.65	24.8	.116	4.4
5.0	.64	24.4	.120	4.6
	.65	24.8	.118	4.5
	.68	26.0	.120	4.6
10.0	.57	21.8	.120	4.6
	.54	20.6	.118	4.5
	.56	21.4	.127	4.8
20.0	.48	18.3	.119	4.5
	.50	19.1	.121	4.6
	.50	19.1	.126	4.8

[a] 1.38 A_{660} units/μmole P_i; Na^+K^+-ATPase S.A. - 21.4.

[b] inhibited with 1 x 10^{-4} M Ouabain.

TABLE 4 . Guinea Pig Kidney ATPase Activity as a Function of KCl Concentration

KCl mM	A_{660}	Total Activity[a]	A_{660}	Mg^{2+} Activity[b]
0.625	.85	22.6	.192	5.1
	.86	22.9	.190	5.0
	.83	22.1	.193	5.1
	.86	22.9	.195	5.2
1.25	.89	23.6	.178	4.7
	.86	22.9	.180	4.8
	.84	22.3	.176	4.7
	.85	22.6	.179	4.8
2.50	.76	20.2	.175	4.6
	.76	20.2	.178	4.7
	.74	19.7	.175	4.6
	.75	19.9	.180	4.8
5.0	.68	18.1	.175	4.6
	.66	17.5	.180	4.8
	.65	17.3	.172	4.6
	.69	18.3	.174	4.6
10.0	.60	16.0	.193	5.1
	.62	16.5	.191	5.1
	.61	16.2	.183	4.9
	.63	16.6	.188	5.0

[a] 1.34 A_{660} units/μmole P_i; Na^+K^+-ATPase S.A. - 18.1.

[b] inhibited with 5×10^{-4} M Ouabain.

TABLE 5 . Inhibition of Guinea Pig Heart ATPase by Various Cardiac Glycosides at Two K^+ Concentrations

Glycoside Molarity	CONVALLATOXOL A_{660}	CONVALLATOXOL % Inhibition	DIGOXIN A_{660}	DIGOXIN % Inhibition	CYMAROL A_{660}	CYMAROL % Inhibition
20.0 mM K^+ [a]						
1×10^{-7}	.306	-36	-	-	.270	- 4
	.315	-44	.345	-70	.270	- 4
5×10^{-7}	.290	-22	.310	-40	.250	13
	.290	-22	.290	-22	.270	- 4
1×10^{-6}	.286	-18	.210	-39	.270	- 4
	.298	-29	.285	-18	.272	- 6
5×10^{-6}	.265	0	.260	4	.245	17
	.260	4	.260	4	.248	15
1×10^{-5}	.230	30	.260	4	.231	30
	.245	17	.185	70	.222	37
5×10^{-5}	.200	56	.195	61	.181	74
	.195	61	.200	56	.190	65
1×10^{-4}	.180	74	.185	70	.175	78
	.180	74	.180	74	.168	84
5×10^{-4}	.160	91	.148	102	.161	90
	.175	78	.148	102	.167	85
0.625 mM K^+ [b]						
1×10^{-7}	.352	13	.303	44	.293	50
	.368	3	.301	45	.284	56
5×10^{-7}	.292	51	.250	78	.251	77
	.306	42	.254	75	.273	63
1×10^{-6}	.260	71	.247	80	.234	88
	.251	77	.251	77	.246	80
5×10^{-6}	.246	80	.238	85	.237	86
	.240	84	.234	88	.239	85
1×10^{-5}	.230	90	.236	87	.233	88
	.236	87	.240	84	.234	88
5×10^{-5}	.262	70	.218	98	.224	94
	.241	83	.218	98	.234	88
1×10^{-4}	.250	78				
	.240	84				
5×10^{-4}	.213	101				
	.226	93				

[a] 1.39 A_{660} units/µmole P_i; Na^+K^+-ATPase S.A. - 7.2; .265 A_{660} in absence of glycoside.

[b] 1.40 A_{660} units/µmole P_i; Na^+K^+-ATPase S.A. - 5.6; .372 A_{660} in absence of glycoside.

TABLE 5 (continued)

Glycoside Molarity	CONVALLATOXOL A_{660}	CONVALLATOXOL % Inhibition	DIGOXIN A_{660}	DIGOXIN % Inhibition	CYMAROL A_{660}	CYMAROL % Inhibition
20.0 mM K^+						
1×10^{-7}	.263	2	.245	17	.292	-24
	.250	13	.245	17	.285	-18
5×10^{-7}	.244	18	.245	17	.248	15
	.258	6	.245	17	.260	4
1×10^{-6}	.237	24	.245	17	.250	13
	.250	13	.235	26	.245	17
5×10^{-6}	.235	26	.212	46	.212	46
	.248	15	.210	48	.202	55
1×10^{-5}	.243	19	.195	61	.200	56
	.234	27	.195	61	.195	61
5×10^{-5}	.195	61	.176	77	.146	104
	.190	65	.157	94	.162	90
1×10^{-4}	.180	74	.146	103	.165	87
	.170	83	.141	108	.140	109
5×10^{-4}	.151	100	.130		.145	
	.150		.130		.147	
0.625 mM K^+						
1×10^{-7}	.303	44	.301	45	.259	72
	.300	46	.309	40	.266	68
5×10^{-7}	.255	74	.251	77	.229	91
	.258	73	.255	74	.228	92
1×10^{-6}	.250	78	.240	84	.225	94
	.242	83	.245	81	.220	97
5×10^{-6}	.265	68	.222	96	.219	97
	.229	91	.229	91	.235	87
1×10^{-5}	.219	97	.231	90	.212	102
	.222	96	.230	90	.222	96
5×10^{-5}	.210		.218	98	.205	106
	.212	100	.228	92	.219	97
1×10^{-4}	.218		.218	98	.214	100
	.217		.215	100	.206	106
5×10^{-4}	.220		.168		.199	
	.230		.160		.199	

TABLE 6 . Inhibition of Guinea Pig Brain ATPase by Various Cardiac Glycosides at 20 mM K^+

Glycoside Molarity	CONVALLATOXOL A_{660}	CONVALLATOXOL % Inhibition	DIGOXIN A_{660}	DIGOXIN % Inhibition	CYMAROL A_{660}	CYMAROL % Inhibition
Study 1[a]						
1×10^{-7}	1.15	- 6	.99	12	.99	12
	1.05	5	1.00	11	.99	12
5×10^{-7}	.94	17	.85	27	.86	26
	.95	16	.87	25	.89	23
1×10^{-6}	.80	33	.85	27	.79	34
	.77	36	.86	26	.76	37
5×10^{-6}	.46	70	.59	56	.46	70
	.44	72	.58	57	.46	70
1×10^{-5}	.324	85	.50	66	.310	87
	.327	85	.48	68	.338	84
5×10^{-5}	.200	99	.250	94	.219	97
	.208	98	.245	94	.204	98
1×10^{-4}	.179	101	.141	106	.142	105
	.184	101	.159	104	.173	102
5×10^{-4}	.186	101				
	.176	102				
Study 2[b]						
1×10^{-7}	.92	7	.92	7	.95	3
	.92	7	.93	6	.95	3
5×10^{-7}	.79	22	.79	22	.83	18
	.80	21	.80	21	.82	19
1×10^{-6}	.65	39	.78	24	.76	26
	.64	40	.78	24	.74	28
5×10^{-6}	.358	74	.54	52	.48	59
	.391	70	.58	47	.48	59
1×10^{-5}	.311	79	.45	63	.361	73
	.293	81	.47	60	.366	73
5×10^{-5}	-	-	.203	92	.198	92
	.180	95	.210	91	.197	93
1×10^{-4}	.146	99	.152	98	.150	98
	.138	100	.147	99	.159	97
5×10^{-4}						

[a] 1.30 A_{660} units/µmole P_i; Na^+K^+-ATPase S.A. - 24.2; 1.10 A_{660} in absence of glycoside.

[b] 1.34 A_{660} units/µmole P_i; Na^+K^+-ATPase S.A. - 21.9; .98 A_{660} in absence of glycoside.

TABLE 6 (continued)

Glycoside Molarity	OUABAIN A_{660}	OUABAIN % Inhibition	DIGITOXIN A_{660}	DIGITOXIN % Inhibition	HELLEBRIN A_{660}	HELLEBRIN % Inhibition
Study 1						
1×10^{-7}	1.10	0	.95	16	.90	22
	1.00	11	.95	16	.90	22
5×10^{-7}	.95	16	.78	35	.68	46
	.98	13	.81	32	.68	46
1×10^{-6}	.92	20	.67	47	.61	54
	.93	18	.69	45	.64	50
5×10^{-6}	.66	48	.354	82	.313	86
	.65	49	.357	82	.320	86
1×10^{-5}	.52	64	.264	92	.317	86
	.51	65	.251	93	.308	87
5×10^{-5}	.289	89	.138	106	.183	101
	.303	88	.140	106	.195	100
1×10^{-4}	.232	96	-	-	.163	103
	.232	96	-	-	.158	104
5×10^{-4}	.189	100				
	.195					
Study 2						
1×10^{-7}	.99	- 1	1.02	- 5	.86	14
	.98	- 2	.97	1	.87	13
5×10^{-7}	.89	9	.80	21	.61	43
	.89	9	.82	19	.61	43
1×10^{-6}	.90	18	.62	42	.56	49
	.85	15	.61	43	.58	47
5×10^{-6}	.53	53	.372	72	.283	82
	.55	50	.359	73	.291	81
1×10^{-5}	.48	59	-	-	.276	83
	.50	56	-	-	.291	81
5×10^{-5}	.250	86	-	-	.156	97
	.252	86	-	-	.159	97
1×10^{-4}	.204	91	.218	90	.141	99
	.202	92	.264	84	.131	100
5×10^{-4}	.131	100	.136	99		
	.132		.130	100		

TABLE 6 (continued)

Glycoside Molarity	A_{660}	% Inhibition	A_{660}	% Inhibition	A_{660}	% Inhibition
Study 3[a]	CONVALLATOXOL		DIGOXIN		CYMAROL	
1×10^{-7}	.73	11	.77	- 1	.72	7
	.74	10	.76	1	.71	8
5×10^{-7}	.68	13	.69	12	.61	24
	.64	20	.69	12	.63	21
1×10^{-6}	.55	34	.65	18	.58	29
	.57	31	.67	15	.60	26
5×10^{-6}	.36	64	.50	42	.42	54
	.35	65	.49	43	.394	58
1×10^{-5}	.268	78	.40	58	.323	70
	.276	77	.381	60	.318	70
5×10^{-5}	.174	93	.196	90	.182	92
	.172	94	.204	89	.182	92
1×10^{-4}	.142	98	.160	96	.155	96
	.136	99	.164	95	.143	98
	OUABAIN		DIGITOXIN		HELLEBRIN	
1×10^{-7}	.76	1	.70	10	.67	15
	.76	1	.66	16	.66	16
5×10^{-7}	.64	20	.59	27	.51	40
	.66	16	.58	29	.51	40
1×10^{-6}	.64	20	.50	42	.46	48
	.64	20	.52	38	.43	53
5×10^{-6}	.45	50	.317	71	.240	83
	.45	50	.307	72	.234	84
1×10^{-5}	.43	53	.230	84	.240	83
	.42	54	.227	85	.232	84
5×10^{-5}	.227	85	.117	102	.158	96
	.225	85	.115	103	.153	97
1×10^{-4}	.175	93	-	-	.126	101
	.183	92	-	-	.128	101
5×10^{-4}	.134	100				
	.132					

[a] 1.35 A_{660} units/μmole P_i; Na^+K^+-ATPase S.A. - 16.4; .76 A_{660} in absence of glycoside.

TABLE 7 . Inhibition of Guinea Pig Brain ATPase by Various Cardiac Glycosides at 0.625 mM K^+

Glycoside Molarity	CONVALLATOXOL A_{660}	CONVALLATOXOL % Inhibition	DIGOXIN A_{660}	DIGOXIN % Inhibition	CYMAROL A_{660}	CYMAROL % Inhibition
Study 1[a]						
1×10^{-8}	.40	52	.310	70	.310	70
	.41	50	.299	72	.323	67
5×10^{-8}	.289	74	.206	91	.210	90
	.290	74	-	-	.221	88
1×10^{-7}	.206	91	.180	96	.202	92
	.201	92	.186	95	.202	92
5×10^{-7}	.180	96	.171	98	.175	98
	.181	96	.167	99	.174	98
1×10^{-6}	.170	98	.159	101	.161	100
	.168	99	.165	100	.168	99
5×10^{-6}	.180	96	.142	104	.159	101
	.172	98	.146	103	.158	101
1×10^{-5}	.165	100	.138	105	.148	103
	.155	102	.142	104	.143	104
5×10^{-5}	.154	102				
	.159	101				
Study 2[b]						
1×10^{-8}	.359	6	.312	21	.326	16
	.352	8	-	-	.317	19
5×10^{-8}	.259	38	.233	46	.253	40
	.273	33	.229	47	.252	40
1×10^{-7}	.209	54	.193	59	.200	57
	.221	50	.191	60	.200	57
5×10^{-7}	.131	79	.111	85	.112	85
	.131	79	.110	85	.121	82
1×10^{-6}	.101	88	.093	91	.092	91
	.103	88	.090	92	.098	89
5×10^{-6}	.084	94	.076	96	.055	103
	.079	95	.066	99	.060	101
1×10^{-5}	.072	98	.067	99	.052	104
	.079	95	.071	98	.057	102
1×10^{-4}	.067	99	.063	100	-	-
	.072	98	.060	101	-	-

[a] 1.34 A_{660} units/µmole P_i; Na^+K^+-ATPase S.A. - 36.0; .66 A_{660} in absence of glycoside.

[b] 1.41 A_{660} units/µmole P_i; Na^+K^+-ATPase S.A. - 23.0; .378 A_{660} in absence of glycoside.

TABLE 7 (continued)

Glycoside Molarity	OUBAIN A_{660}	OUBAIN % Inhibition	DIGITOXIN A_{660}	DIGITOXIN % Inhibition	HELLEBRIN A_{660}	HELLEBRIN % Inhibition
Study 1						
1×10^{-8}	.371	58	.298	72	.277	77
	.285	55	.295	73	.269	78
5×10^{-8}	.255	81	.201	92	.192	94
	.261	80	.202	92	.198	93
1×10^{-7}	.282	76	.182	96	.198	93
	.230	86	.190	94	.190	94
5×10^{-7}	.185	96	.155	102	.166	99
	.190	94	.160	101	.165	100
1×10^{-6}	.175	98	.148	103	.170	98
	-	-	.151	102	.168	99
5×10^{-6}	-	-	-	-	.161	100
	-	-	-	-	.163	100
1×10^{-5}	-	-	-	-	.161	100
	.166	99	.149	103	.154	102
5×10^{-5}	.162	100				
	.163					
Study 2						
1×10^{-8}	.336	13	.327	16	.280	31
	.334	14	.342	11	.291	28
5×10^{-8}	.287	29	.218	51	.162	69
	.277	32	.213	52	.172	66
1×10^{-7}	.252	40	.203	56	.132	78
	.247	42	.193	59	.136	77
5×10^{-7}	.156	71	.107	86	.090	92
	.176	64	.108	86	.088	92
1×10^{-6}	.125	80	.096	90	.082	94
	.120	82	.097	90	.083	94
5×10^{-6}	.077	96	.067	99	.060	101
	.082	94	.059	102	.061	101
1×10^{-5}	.076	96	-	-	.062	101
	.078	96	-	-	.062	101
1×10^{-4}	.066	100				
	.065					

TABLE 7 (continued)

Glycoside Molarity	A_{660}	% Inhibition	A_{660}	% Inhibition	A_{660}	% Inhibition
Study 3[c]	CONVALLATOXOL		DIGOXIN		CYMAROL	
1×10^{-8}	.261	6	.283	- 4	.249	11
	.260	6	.260	6	.241	15
5×10^{-8}	.200	33	.230	20	.201	32
	.200	33	.232	19	.203	32
1×10^{-7}	.171	46	.221	24	.179	42
	.163	49	.206	30	.174	44
5×10^{-7}	.098	78	.221	24	.114	71
	.092	81	.231	19	.110	73
1×10^{-6}	.080	86	.097	78	.087	83
	.074	88	.091	81	.091	81
5×10^{-6}	.058	96	.070	90	.072	89
	.059	95	.062	94	.061	94
1×10^{-5}	.054	97	.060	95	.061	94
	.054	97	.061	94	.060	95
5×10^{-5}	.050	99	.050	99	.051	99
	.051	99	.050	99	.051	99
5×10^{-4}	.050	99				
	.052	98				
	OUBAIN		DIGITOXIN		HELLEBRIN	
1×10^{-8}	.257	8	.261	6	.238	16
	.257	8	.236	17	.247	12
5×10^{-8}	.235	17	.190	37	.180	42
	.230	20	.157	52	.181	41
1×10^{-7}	.200	33	.157	52	.156	52
	.198	34	.161	50	.160	50
5×10^{-7}	.127	65	.081	85	.102	76
	.123	67	.091	81	.100	77
1×10^{-6}	.099	78	.081	85	.082	85
	.107	74	.081	85	.082	85
5×10^{-6}	.068	91	.061	94	.060	95
	.070	90	.061	94	.068	91
1×10^{-5}	.067	92	.050	99	.053	98
	.073	89	.053	98	.056	96
5×10^{-5}	.057	96	.042	103	.049	100
	.050	99	.050	99	.057	96
5×10^{-4}	.047	100				
	.049					

[c] 1.41 A_{660} units/µmole P_i; Na^+K^+-ATPase S.A. - 18.4; .304 A_{660} in absence of glycoside.

TABLE 8 . Inhibition of Guinea Pig Kidney ATPase by Various Cardiac Glycosides at 20 mM K^+

Glycoside Molarity	CONVALLATOXOL A_{660}	CONVALLATOXOL % Inhibition	DIGOXIN A_{660}	DIGOXIN % Inhibition	CYMAROL A_{660}	CYMAROL % Inhibition
Study 1[a]						
1×10^{-7}	.48	- 4	.50	-12	.46	3
	.50	-12	.52	-20	.48	- 4
5×10^{-7}	.48	- 4	.44	- 8	.45	7
	.49	- 8	.52	-20	.43	15
1×10^{-6}	.44	9	.48	- 4	.42	18
	.45	7	.47	0	.45	7
5×10^{-6}	.332	52	.393	29	.333	52
	.360	41	.387	31	.335	51
1×10^{-5}	.288	69	.337	50	.281	71
	.297	65	.351	45	.290	68
5×10^{-5}	.230	91	.225	93	.216	96
	.231	90	.220	95	.225	93
1×10^{-4}	.217	96	.190	106	.197	103
	.230	91	-		.204	101
5×10^{-4}	.198	103				
	.183	109				
Study 2[b]						
1×10^{-7}	.51	7	.53	1	.51	7
	.55	- 4	.53	1	.53	1
5×10^{-7}	.51	7	.53	1	.46	21
	.52	4	.54	- 2	.44	26
1×10^{-6}	.45	24	.56	- 7	.45	24
	.48	15	.52	4	.45	24
5×10^{-6}	.376	44	.41	35	.363	48
	.360	49	.41	35	.358	50
1×10^{-5}	.295	67	.369	46	.310	63
	.298	66	.356	50	.298	66
5×10^{-5}	.228	86	.223	88	.216	90
	.224	87	.215	90	.208	92
1×10^{-4}	.210	91	.184	98	.187	98
	.202	94	.181	99	.185	98
5×10^{-4}	.181	99				
	.173	102				

[a] 1.39 A_{660} units/µmole P_i; Na^+K^+-ATPase S.A. - 6.9; .47 A_{660} in absence of glycoside.

[b] 1.36 A_{660} units/µmole P_i; Na^+K^+-ATPase S.A. - 9.3; .53 A_{660} in absence of glycoside.

TABLE 8 (continued)

Glycoside Molarity	CONVALLATOXOL A_{660}	CONVALLATOXOL % Inhibition	DIGOXIN A_{660}	DIGOXIN % Inhibition	CYMAROL A_{660}	CYMAROL % Inhibition
Study 1						
1×10^{-7}	.49	- 8	.45	7	.44	11
	.51	-16	.48	- 4	.44	11
5×10^{-7}	.50	-12	.43	15	.42	18
	.49	- 8	.44	11	.40	26
1×10^{-6}	.48	- 4	.389	30	.355	43
	.48	- 4	.39	30	.386	31
5×10^{-6}	.41	22	.289	68	.280	72
	.41	22	.269	76	.263	78
1×10^{-5}	.362	39	.144	124	.283	71
	.366	39	.147	122	.281	71
5×10^{-5}	.269	76	-	-	.213	97
	.262	79	-	-	.216	96
1×10^{-4}	.230	91	.221	94	.203	101
	.237	88	-	-	.221	94
5×10^{-4}	.205	100				
	.207					
Study 2						
1×10^{-7}	.50	10	.52	4	.48	15
	.49	12	.52	4	.50	10
5×10^{-7}	.48	15	.48	15	.50	10
	.48	15	.48	15	.41	35
1×10^{-6}	.43	29	-	-	.375	45
	.47	18	.43	29	.398	38
5×10^{-6}	.40	38	.307	64	.254	79
	.41	35	.304	65	.260	78
1×10^{-5}	.354	51	.249	80	.290	69
	.362	48	.250	80	.282	71
5×10^{-5}	.234	84	.158	106	.200	94
	.242	82	.151	108	.201	94
1×10^{-4}	.217	89	-		.190	97
	.212	91	-		.182	99
5×10^{-4}	.182	100				
	.176					

TABLE 9. Inhibition of Guinea Pig Kidney ATPase by Various Cardiac Glycosides at 0.625 mM K^+. [a]

Glycoside Molarity	A_{660}	% Inhibition	A_{660}	% Inhibition	A_{660}	% Inhibition
	CONVALLATOXOL		DIGOXIN		CYMAROL	
1×10^{-8}	.65	11	.51	38	.49	42
	.65	11	.54	32	.48	44
5×10^{-8}	.60	21	.316	75	.304	77
	.62	17	.320	74	.289	80
1×10^{-7}	.64	13	.275	83	.269	84
	.68	5	.280	82	.259	86
5×10^{-7}	.51	38	.210	95	.194	98
	.50	40	.211	95	.204	96
1×10^{-6}	.254	87	.195	98	.179	101
	.256	87	.190	99	.197	98
5×10^{-6}	.210	95	.185	100	.184	100
	.208	96	.190	99	.179	101
1×10^{-5}	.183	101	.168	103	.179	101
	.191	99	.180	101	.183	101
5×10^{-5}	-	-				
	.202	97				
5×10^{-4}	.180	101				
	.181	101				
	OUABAIN		DIGITOXIN		HELLEBRIN	
1×10^{-7}	.62	17	.46	48	.303	78
	.60	21	.393	60	.303	78
5×10^{-7}	.40	59	.241	89	.294	79
	.41	57	.233	91	.295	79
1×10^{-6}	.334	72	.205	96	.175	102
	.319	74	.213	95	.181	101
5×10^{-6}	.214	95	.174	102	.163	104
	.224	93	.172	103	.173	102
1×10^{-5}	.197	98	.157	106	.173	102
	.217	94	.163	104	.163	104
5×10^{-5}	.197	98	-	-	.155	106
	.204	96	-	-	.154	106
1×10^{-4}	.184	100				
	.199	98				
5×10^{-4}	.189	100				
	.186					

[a] 1.30 A_{660} units/μmole P_i; Na^+K^+-ATPase S.A. - 14.4; A_{660} in absence of glycoside.

TABLE 10. Experimental data for the influence of various ligands on the binding of cardiac glycosides by guinea pig Na^+K^+-ATPase

Glycoside Molarity	bound	unbound	b/u	bound	unbound	b/u
			KIDNEY			
	without ATP[a]			with 20 mM K^+[a]		
1×10^{-8}	0	11.9	-	0.101	12.0	0.008
	0	12.0	-	0.101	11.6	0.009
1×10^{-7}	0.229	113.	0.002	1.42	107.	0.013
	0.070	110.	0.001	1.34	111.	0.012
1×10^{-6}	0.199	1060.	0.000	14.6	1164.	0.012
	0	1108.	-	18.7	1164.	0.016
			HEART			
	without ATP[a]			with 20 mM K^+[a]		
1×10^{-8}	0.042	7.24	0.006	0.54	10.3	0.052
	0.036	7.12	0.005	1.06	9.92	0.107
3×10^{-8}	0.079	22.1	0.004	1.43	31.2	0.046
	0.006	21.6	0.000	1.18	31.8	0.037
1×10^{-7}	0.192	75.2	0.002	3.86	96.0	0.040
	0.240	80.0	0.003	3.69	98.4	0.038
3×10^{-7}	0.528	245.	0.002	7.66	310.	0.025
	0.024	240.	0.000	8.03	318.	0.025
	without Na[a]			with 20 mM K^+[b]		
1×10^{-8}	2.09	6.56	0.318	0.28	9.00	0.031
	2.03	6.44	0.315	0.25	9.08	0.028
3×10^{-8}	4.95	20.4	0.243	0.73	29.3	0.025
	5.14	20.9	0.246	0.84	28.6	0.029
1×10^{-7}	13.5	75.2	0.180	2.00	87.2	0.023
	14.0	78.4	0.178	2.09	91.2	0.023
3×10^{-7}	20.1	254.	0.079	5.88	286.	0.020
	20.9	251.	0.083	7.50	284.	0.026

[a] convallatoxol

[b] ouabain

TABLE10(continued)

Glycoside Molarity	bound	unbound	b/u	bound	unbound	b/u
			BRAIN			
	without ATP[a]			with 20 mM K^+[a]		
1×10^{-8}	.098	9.30	0.010	2.14	7.36	0.291
	.057	9.33	0.006	2.00	7.47	0.268
1×10^{-7}	.988	81.6	0.012	15.3	70.2	0.218
	1.35	87.0	0.016	15.1	70.6	0.214
1×10^{-6}	11.8	873.	0.014	74.3	797.	0.093
	9.88	853.	0.012	74.9	809.	0.092
	without ATP[b]			with 20 mM K^+[a]		
1×10^{-9}	.019	1.14	0.017	.213	1.03	0.207
	.018	1.29	0.014	.112	1.06	0.106
3×10^{-9}	.049	3.93	0.012	.383	3.20	0.120
	.048	3.99	0.012	.353	3.29	0.107
1×10^{-8}	.130	14.2	0.009			
	.142	14.1	0.010			

[a] convallatoxol

[b] digoxin

TABLE 11. Experimental binding data for guinea pig kidney Na^+K^+-ATPase at various enzyme concentrations at fixed glycoside concentration

µl Enzyme Solution[a]	bound	unbound	b/u	bound	unbound	b/u
	CONVALLATOXOL[b]			DIGOXIN[b]		
25	4.7	6.48	0.725	2.2	7.72	0.285
	4.9	6.56	0.747	2.1	7.72	0.272
50	6.6	4.64	1.42	3.6	6.56	0.549
	6.7	4.92	1.36	3.3	6.56	0.503
100	7.9	3.16	2.50	4.5	3.40	1.32
	6.9	3.52	1.96	4.9	3.40	1.44
150	8.2	2.96	2.77	5.1	3.24	1.57
	8.0	2.80	2.86	4.9	2.80	1.75
	OUABAIN[b]					
25	2.1	8.24	0.255			
	2.1	8.24	0.255			
50	3.8	6.84	0.556			
	3.8	7.08	0.537			
100	5.4	5.36	1.01			
	5.1	5.44	0.938			
150	5.4	4.80	1.12			
	5.5	5.08	1.08			

[a] mg protein/50 µl.

[b] 1×10^{-8} M

TABLE 12. Experimental binding data for guinea pig brain Na^+K^+-ATPase at various enzyme concentrations at fixed glycoside concentration

µl Enzyme Solution[a]	bound	unbound	b/u	bound	unbound	b/u
CONVALLATOXOL[b]				CYMAROL		
25	9.2	1.79	5.14	7.61	2.93	2.60
	9.4	1.51	6.22	7.06	2.80	2.52
50	10.0	0.645	15.5	9.22	1.20	7.68
	10.5	0.681	15.4	8.77	1.35	6.50
100	10.5	0.286	36.7	9.85	0.922	10.7
	10.4	0.265	39.2	9.76	0.666	14.6
150	10.6	0.348	30.4			
	10.5	0.338	31.1			
DIGOXIN[b]						
25	4.8	3.55	1.35	6.34	5.05	1.26
	4.5	3.92	1.15	5.89	5.62	1.05
50	6.5	2.62	2.48	7.99	3.92	2.04
	6.7	2.75	2.44	8.22	4.90	1.68
100	7.0	1.92	3.64	10.4	1.86	5.59
	7.3	2.11	3.46	9.95	2.28	4.36
150	7.5	1.78	4.21			
	7.2	1.77	4.07			
OUABAIN[b]						
25	5.4	3.71	1.46	5.16	5.84	0.884
	5.4	3.90	1.38	4.81	5.75	0.836
50	7.4	2.42	3.06	7.01	4.23	1.66
	7.5	2.55	2.94	6.69	4.24	1.58
100	9.0	1.47	6.12	8.44	2.22	3.80
	8.5	1.50	5.67	8.46	2.14	3.95
150	9.0	1.18	7.63			
	8.7	1.23	7.07			

[a] mg protein/50 µl
[b] 1×10^{-8} M

TABLE 13. Experimental data for guinea pig heart Na^+K^+-ATPase/convallatoxol binding in the presence of various concentrations of K^+

Glycoside Molarity	bound	unbound	b/u	bound	unbound	b/u
	0 K^+			0.155 mM K^+		
1×10^{-8}	5.46	7.28	0.750	5.02	8.36	0.600
	5.41	7.08	0.764	3.75	8.64	0.434
3×10^{-8}	14.4	23.8	0.605	11.8	25.8	0.457
	13.4	24.1	0.556	12.5	25.0	0.500
1×10^{-7}	26.4	85.2	0.310	23.5	94.0	0.250
	28.5	88.8	0.321	25.2	95.6	0.264
3×10^{-7}	39.8	315.	0.126	33.9	310.	0.109
	39.2	316.	0.124	35.5	320.	0.111
1×10^{-6}	45.8	1072.	0.043	44.4	1148.	0.039
	46.5	1064.	0.044	43.5	1168.	0.037
3×10^{-6}	52.6	3376.	0.016	46.5	3288.	0.014
	42.4	3424.	0.012	57.0	3380.	0.017
1×10^{-5}	72.7	10688.	0.007	67.1	11432.	0.006
	61.0	11136.	0.005	111.	11744.	0.009
3×10^{-5}	-	-	-	-	-	-
	1.4	35840.	0.000	-	-	-
	0.625 mM K^+			20 mM K^+		
1×10^{-8}	2.80	9.00	0.311	.23	9.04	0.025
	2.69	9.12	0.295	.25	10.1	0.025
3×10^{-8}	6.89	29.2	0.236	.78	36.0	0.022
	7.15	30.4	0.235	.72	35.5	0.020
1×10^{-7}	11.9	96.4	0.123	1.87	92.8	0.020
	14.3	101.	0.142	1.66	94.0	0.018
3×10^{-7}	21.8	336.	0.065	5.38	343.	0.016
	22.1	337.	0.066	5.08	350.	0.014
1×10^{-6}	31.3	1112.	0.028	8.21	896.	0.009
	35.3	936.	0.038	10.5	932.	0.011
3×10^{-6}	43.9	3376.	0.013	72.6	3512.	0.021
	19.8	3468.	0.006	-	3484.	-
1×10^{-5}	73.7	10448.	0.007	17.0	9152.	0.002
	49.7	11064.	0.004	12.5	9604.	0.001
3×10^{-5}	-	34696.	-	65.6	35740.	0.002
	-	35812.	-	-	36120.	-

TABLE 14. Experimental data for guinea pig brain Na^+K^+-ATPase/convallatoxol binding in the presence of various K^+ concentrations

Glycoside Molarity	bound	unbound	b/u	bound	unbound	b/u
		0 K^+			0.155 mM K^+	
1×10^{-8}	27.3	1.44	19.0	27.5	1.68	16.4
	25.8	1.56	16.5	27.3	1.60	17.1
3×10^{-8}	76.3	8.04	9.49	73.5	7.72	9.52
	77.5	7.28	10.6	77.3	7.40	10.4
1×10^{-7}	143.	59.6	2.40	135.	61.2	2.20
	152.	59.6	2.55	137.	65.2	2.10
3×10^{-7}	179.	282.	0.635	171.	273.	0.626
	176.	292.	0.603	174.	291.	0.598
1×10^{-6}	148.	1012.	0.146	202.	996.	0.203
	211	1032.	0.204	199.	1016.	0.196
3×10^{-6}	272.	3276.	0.083	227.	3312.	0.068
	259.	3316.	0.078	236.	3316.	0.071
1×10^{-5}	248.	11000.	0.022	204.	11032.	0.018
	291.	11364.	0.026	249.	11704.	0.021
3×10^{-5}	243.	33752.	0.007	491.	33308.	0.015
	172.	35380.	0.005	582.	34612.	0.017
		0.625 mM K^+			20.0 mM K^+	
1×10^{-8}	25.6	2.32	11.0	6.78	9.12	0.743
	25.4	2.16	11.8	7.32	8.96	0.817
3×10^{-8}	70.3	10.6	6.63	18.8	28.1	0.669
	67.0	10.0	6.70	18.3	28.4	0.644
1×10^{-7}	129.	64.0	2.02	40.0	97.2	0.412
	132.	67.6	1.95	45.7	91.2	0.501
3×10^{-7}	157.	292.	0.538	78.8	307.	0.257
	153.	294.	0.520	78.8	324.	0.243
1×10^{-6}	196.	1036.	0.189	129.	1076.	0.120
	186.	1092.	0.170	136.	1020.	0.133
3×10^{-6}	218.	3232.	0.067	181.	3408.	0.053
	221.	3268.	0.068	222.	3292.	0.067
1×10^{-5}	263.	11304.	0.023	500.	11088.	0.045
	381.	11080.	0.034	360.	11492.	0.031
3×10^{-5}	867.	33732.	0.026	279.	34824.	0.008
	560.	33552.	0.017	442.	33264.	0.013

TABLE 14. (continued)

Glycoside Molaridy	bound	unbound	b/u	bound	unbound	b/u
		0 K^+			0.625 mM K^+	
1×10^{-8}	20.6	1.09	18.9	17.2	1.30	13.2
	19.6	1.16	17.0	17.0	1.35	12.7
3×10^{-8}	55.4	3.73	14.9	46.7	4.38	10.7
	50.9	4.06	12.6	48.7	4.23	11.5
1×10^{-7}	106.	19.7	5.40	91.8	20.8	4.46
	110.	19.4	5.68	92.7	20.7	4.46
3×10^{-7}	149.	71.7	2.03	133.	73.0	1.76
	145.	72.0	2.03	132.	73.0	1.76
		1.25 mM K^+			2.50 mM K^+	
1×10^{-8}	15.4	1.45	10.6	11.6	1.76	6.59
	15.0	1.50	10.0	11.2	1.78	6.29
3×10^{-8}	38.2	5.01	7.62	30.5	5.58	5.46
	41.3	4.78	8.64	32.1	5.46	5.88
1×10^{-7}	88.4	21.1	4.19	63.0	22.9	2.75
	87.6	21.1	4.15	64.6	22.8	2.83
3×10^{-7}	128.	73.3	1.75	110.	74.7	1.47
	118.	74.0	1.59	108.	74.8	1.44

These values were derived from (total pmoles present-pmoles bound). This method is appropriate since in other experiments the total pmoles added could be accounted for by pmoles bound + pmoles unbound within reasonable limits.

TABLE 15. Experimental binding data for guinea pig brain Na^+K^+-ATPase in the absence of K^+

Glycoside Molarity	bound	unbound	b/u	bound	unbound	b/u
		CONVALLATOXOL			CYMAROL	
1×10^{-8}	15.4	0.96	16.0	12.7	1.21	10.5
	15.6	0.96	16.2	13.1	1.35	9.70
3×10^{-8}	48.0	4.06	11.8	38.7	5.02	7.71
	47.1	4.13	11.4	40.8	5.20	7.85
5×10^{-8}	75.2	8.86	8.49	63.6	9.24	6.88
	72.4	9.95	7.28	65.1	10.0	6.51
1×10^{-7}	108.	32.3	3.34	108.	33.5	3.22
	110.	36.1	3.05	109.	32.8	3.32
		DIGOXIN			DIGITOXIN	
1×10^{-8}	11.3	3.52	3.21	9.08	1.28	7.09
	10.6	3.57	2.97	8.16	1.42	5.75
3×10^{-8}	34.4	12.6	2.73	23.4	4.65	5.56
	33.9	13.3	2.55	27.7	4.21	6.58
5×10^{-8}	52.5	22.8	2.30	43.2	7.28	5.93
	52.4	22.8	2.30	45.6	6.85	6.66
1×10^{-7}	77.3	48.8	1.58	84.0	16.4	5.12
	74.5	47.6	1.56	83.2	17.8	4.67
		OUABAIN				
1×10^{-8}	14.4	4.51	3.19			
	14.2	4.74	3.00			
3×10^{-8}	39.6	16.4	2.41			
	40.2	16.0	2.51			
5×10^{-8}	58.9	31.2	1.89			
	62.8	27.4	2.29			
1×10^{-7}	91.4	54.4	1.68			
	87.1	57.1	1.52			

TABLE 16. Experimental binding data for guinea pig brain Na^+K^+-ATPase in the absence of K^+

Glycoside Molarity	bound	unbound	b/u	bound	unbound	b/u
	CONVALLATOXOL			CYMAROL		
1×10^{-8}	9.3	0.72	12.9	8.4	1.00	8.40
	9.8	0.84	11.7	8.3	1.08	7.68
3×10^{-8}	40.0	2.88	13.9	27.2	3.76	7.23
	30.5	3.04	10.0	25.2	3.60	7.00
1×10^{-7}	76.1	14.5	5.25	74.0	21.9	3.38
	81.3	18.8	4.32	83.4	23.1	3.61
3×10^{-7}	164.	134.	1.22	164.	153.	1.07
	160.	140.	1.14	161.	167.	0.964
	DIGOXIN			DIGITOXIN		
1×10^{-8}	6.1	2.44	2.50	4.6	0.84	5.48
	6.2	2.72	2.28	4.9	0.88	5.57
3×10^{-8}	18.0	9.28	1.94	14.2	2.88	4.93
	18.2	8.92	2.04	14.9	2.96	5.03
1×10^{-7}	57.6	32.8	1.76	46.5	12.0	3.86
	58.2	39.4	1.48	49.2	12.7	3.87
3×10^{-7}	179.	94.4	1.90	93.1	98.0	0.950
	125.	162.	0.772	52.0	154.	0.338
	OUABAIN					
1×10^{-8}	7.2	2.44	2.95			
	7.3	2.60	2.81			
3×10^{-8}	21.7	9.00	2.41			
	22.4	9.00	2.49			
1×10^{-7}	60.2	31.0	1.94			
	60.2	35.2	1.71			
3×10^{-7}	149.	148.	1.01			
	-	-	-			

TABLE 17. Experimental binding data for guinea pig brain Na^+K^+-ATPase in the absence of K^+

Glycoside Molarity	bound	unbound	b/u	bound	unbound	b/u
	CONVALLATOXOL			CYMAROL		
1×10^{-8}	15.6	0.982	15.9	13.1	1.30	10.1
	15.7	1.08	14.5	12.7	1.42	8.94
3×10^{-8}	47.5	4.24	11.2	40.2	5.08	7.91
	43.2	4.34	9.95	39.4	5.50	7.16
1×10^{-7}	112.	32.0	3.50	114.	31.9	3.57
	114.	33.7	3.38	113.	32.2	3.51
3×10^{-7}	165.	210.	0.786	188.	192.	0.979
	159.	220.	0.723	186.	203.	0.916
	DIGOXIN			DIGITOXIN		
1×10^{-8}	9.78	4.10	2.38	8.43	1.27	6.64
	9.74	4.22	2.31	9.08	1.28	7.09
3×10^{-8}	28.7	14.0	2.05	24.0	4.44	5.40
	28.9	14.3	2.02	24.0	3.47	6.92
1×10^{-7}	72.2	53.9	1.34	78.7	15.4	5.11
	72.2	53.9	1.34	83.3	17.4	4.79
3×10^{-7}	126.	226.	0.558	175.	94.6	1.85
	121.	237.	0.510	170.	108.	1.57
	OUABAIN					
1×10^{-8}	12.8	5.14	2.49			
	12.3	5.11	2.41			
3×10^{-8}	39.6	17.3	2.29			
	36.7	17.6	2.08			
1×10^{-7}	84.9	55.7	1.52			
	87.4	58.5	1.49			
3×10^{-7}	157.	251.	0.625			
	153.	251.	0.610			

TABLE 18. Experimental binding data for guinea pig brain Na^+K^+-ATPase in the presence of 0.625 mM K^+

Glycoside Molarity	bound	unbound	b/u	bound	unbound	b/u
	CONVALLATOXOL			CYMAROL		
1×10^{-8}	13.3	2.11	6.30	11.0	2.61	4.21
	14.3	2.05	6.98	11.0	2.86	3.85
3×10^{-8}	40.5	8.51	4.76	31.5	9.79	3.22
	40.2	8.67	4.64	32.1	9.87	3.25
5×10^{-8}	52.3	12.6	4.15	51.8	16.6	3.12
	54.3	13.4	4.05	50.9	17.6	2.89
1×10^{-7}	97.8	47.1	2.08	60.0	32.6	1.84
	90.5	47.3	1.91	62.4	30.8	2.02
	DIGOXON			DIGITOXIN		
1×10^{-8}	9.48	5.42	1.75	7.68	1.43	5.37
	8.66	5.35	1.62	6.95	1.41	4.93
3×10^{-8}	25.1	16.7	1.50	25.1	4.77	5.26
	24.9	17.2	1.45	25.3	5.02	5.04
5×10^{-8}	39.1	30.9	1.26	43.7	8.64	5.06
	41.0	32.0	1.28	44.3	8.90	4.98
1×10^{-7}	60.1	63.2	0.951	61.8	19.2	3.22
	58.1	66.6	0.872	77.0	21.3	3.62
	OUABAIN					
1×10^{-8}	9.48	7.50	1.26			
	9.21	7.45	1.24			
3×10^{-8}	27.3	25.4	1.07			
	28.5	23.9	1.19			
5×10^{-8}	44.0	41.2	1.07			
	45.1	40.5	1.11			
1×10^{-7}	63.5	75.0	0.847			
	64.4	71.2	0.904			

TABLE 19. Experimental binding data for guinea pig brain Na^+K^+-ATPase in the presence of 0.625 mM K^+

Glycoside Molarity	bound	unbound	b/u	bound	unbound	b/u
	CONVALLATOXOL			CYMAROL		
1×10^{-8}	13.8	2.05	6.73	11.3	2.41	4.69
	13.1	2.00	6.55	11.8	2.41	4.90
3×10^{-8}	42.6	7.21	5.91	35.2	8.41	4.18
	38.7	7.51	5.15	34.5	8.79	3.92
1×10^{-7}	93.7	35.8	2.62	88.6	42.8	2.07
	-	-	-	91.1	45.1	2.02
3×10^{-7}	140.	231.	0.606	143.	226.	0.633
	150.	229.	0.655	147.	223.	0.659
	DIGOXIN			DIGITOXIN		
1×10^{-8}	8.49	4.89	1.74	8.79	1.46	6.02
	7.97	5.18	1.54	7.59	1.31	5.79
3×10^{-8}	26.2	16.9	1.55	23.5	4.52	5.20
	27.3	16.8	1.62	23.1	4.70	4.91
1×10^{-7}	54.3	62.3	0.872	67.9	24.4	2.78
	61.5	63.3	0.972	80.0	24.0	3.33
3×10^{-7}	118.	237.	0.498	143.	93.1	1.54
	117.	244.	0.480	138.	138.	1.00
1×10^{-8}	9.6	7.32	1.03			
	8.9	7.16	1.24			
3×10^{-8}	29.1	23.7	1.23			
	28.6	24.2	1.18			
1×10^{-7}	63.0	70.6	0.892			
	62.8	72.6	0.865			
3×10^{-7}	115.	270.	0.426			
	114.	272.	0.419			

TABLE 20. Experimental binding data for guinea pig brain Na^+K^+-ATPase in the presence of 0.625 mM K^+

Glycoside Molarity	bound	unbound	b/u	bound	unbound	b/u
		CONVALLATOXOL			CYMAROL	
1×10^{-8}	21.7	4.04	5.37	18.3	3.72	4.92
	21.5	3.60	5.97	17.5	3.68	4.76
3×10^{-8}	60.8	14.8	4.11	46.0	15.5	2.97
	57.5	18.8	3.06	48.0	15.1	3.18
1×10^{-7}	102.	74.4	1.37	106.	65.6	1.62
	95.1	80.0	1.19	97.4	73.6	1.32
3×10^{-7}	136.	313.	0.43	140.	286.	0.490
	138.	321.	0.430	146.	292.	0.500
1×10^{-6}	184.	1100.	0.167	296.	1000.	0.296
	160.	1120.	0.143	294.	984.	0.299
3×10^{-6}	189.	4252.	0.044	404.	3272.	0.123
	142.	3600.	0.039	347.	3384.	0.102
		DIGOXIN			DIGITOXIN	
1×10^{-8}	11.7	8.00	1.46	16.3	2.28	7.15
	11.8	8.00	1.48	17.1	2.56	6.68
3×10^{-8}	35.4	25.3	1.40	45.8	9.52	4.81
	33.1	26.2	1.26	44.0	9.12	4.82
1×10^{-7}	66.3	82.8	0.801	108.	50.0	2.16
	63.2	88.0	0.718	97.2	49.6	1.96
3×10^{-7}	122.	312.	0.391	143.	211.	0.678
	103.	322.	0.320	134.	213.	0.629
1×10^{-6}	164.	1064.	0.154	199.	828.	0.240
	186.	1056.	0.176	212.	816.	0.260
3×10^{-6}	160.	3544.	0.045	280.	2616.	0.107
	201.	3528.	0.057	224.	2568.	0.087
		OUABAIN				
1×10^{-8}	14.9	7.96	1.87			
	12.6	8.68	1.45			
3×10^{-8}	36.5	27.5	1.33			
	33.1	28.2	1.17			
1×10^{-7}	19.6	126.	0.156			
	19.1	130.	0.147			
3×10^{-7}	20.2	412.	0.049			
	20.7	424.	0.049			
1×10^{-6}	28.6	1376.	0.021			
	37.4	1352.	0.028			
3×10^{-6}	46.1	4408.	0.010			
	47.3	4224.	0.011			

TABLE 21. Experimental binding data for guinea pig kidney Na^+K^+-ATPase in the absence of K^+

Glycoside Molarity	bound	unbound	b/u	bound	unbound	b/u
		CONVALLATOXOL			CYMAROL	
1×10^{-8}	5.7	5.44	1.05	5.7	3.32	1.72
	6.1	5.52	1.10	5.8	4.48	1.29
3×10^{-8}	17.7	17.5	1.01	16.6	14.4	1.15
	17.7	17.8	0.994	16.7	14.7	1.14
1×10^{-7}	47.4	66.0	0.718	48.4	64.0	0.756
	43.1	60.8	0.709	47.4	67.6	0.701
3×10^{-7}	73.7	258.	0.286	79.6	267.	0.298
	72.1	266.	0.271	80.0	272.	0.294
		DIGOXIN			DIGITOXIN	
1×10^{-8}	2.9	6.24	0.465	2.7	2.44	1.11
	2.8	6.48	0.432	2.9	2.64	1.10
3×10^{-8}	9.2	20.5	0.449	8.9	8.40	1.06
	9.0	20.8	0.433	9.2	8.48	1.08
1×10^{-7}	25.1	70.4	0.356	28.0	31.4	0.892
	25.6	76.8	0.333	30.7	33.3	0.922
3×10^{-7}	46.0	276.	0.167	60.2	155.	0.388
	47.4	281.	0.169	54.7	153.	0.358
		OUABAIN				
1×10^{-8}	3.8	6.64	0.572			
	3.8	6.84	0.556			
3×10^{-8}	12.1	23.1	0.524			
	11.9	23.2	0.513			
1×10^{-7}	29.2	72.0	0.406			
	28.7	73.2	0.392			
3×10^{-7}	61.6	270.	0.228			
	62.7	269.	0.233			

TABLE 22. Experimental binding data for guinea pig kidney Na^+K^+-ATPase in the absence of K^+

Glycoside Molarity	bound	unbound	b/u	bound	unbound	b/u
		CONVALLATOXOL			CYMAROL	
1×10^{-8}	6.04	5.00	1.21	6.04	4.32	1.40
	6.13	4.76	1.29	5.66	4.92	1.15
3×10^{-8}	19.4	11.1	1.75	18.3	14.9	1.23
	19.1	17.0	1.12	15.6	14.4	1.08
1×10^{-7}	44.9	56.0	0.802	51.9	54.4	0.954
	41.3	58.4	0.707	50.9	61.6	0.826
3×10^{-7}	79.2	259.	0.306	78.9	278.	0.284
	82.2	258.	0.319	78.8	278.	0.283
		DIGOXIN			DIGITOXIN	
1×10^{-8}	2.64	6.20	0.426	2.87	2.44	1.18
	2.92	5.92	0.493	2.87	2.28	1.26
3×10^{-8}	8.68	20.2	0.430	9.24	8.08	1.14
	8.44	19.6	0.431	9.74	8.48	1.15
1×10^{-7}	27.1	71.2	0.381	27.6	31.4	0.879
	28.6	68.4	0.418	28.9	30.9	0.935
3×10^{-7}	57.0	263.	0.217	59.7	159.	0.375
	46.2	263.	0.176	59.6	162.	0.368
		OUABAIN				
1×10^{-8}	3.85	7.00	0.550			
	3.70	6.96	0.532			
3×10^{-8}	11.8	23.1	0.511			
	11.7	23.3	0.502			
1×10^{-7}	30.3	73.2	0.414			
	30.2	70.4	0.429			
3×10^{-7}	68.6	275.	0.249			
	69.3	272.	0.255			

TABLE 23. Experimental binding data for guinea pig kidney Na^+K^+-ATPase in the presence of 0.625 mM K^+

Glycoside Molarity	bound	unbound	b/u	bound	unbound	b/u
		CONVALLATOXOL			CYMAROL	
1×10^{-8}	3.73	7.78	0.479	3.43	7.41	0.463
	3.76	7.77	0.484	3.58	7.09	0.505
3×10^{-8}	10.9	24.2	0.450	10.3	22.1	0.466
	10.8	24.1	0.448	10.3	23.0	0.448
1×10^{-7}	30.3	81.9	0.370	28.9	84.4	0.342
	29.2	88.1	0.331	33.1	81.3	0.407
3×10^{-7}	63.2	290.	0.218	54.8	298.	0.184
	59.8	297.	0.201	57.9	317.	0.183
1×10^{-6}	80.5	1056.	0.076	-	1135.	-
	94.8	1063.	0.089	58.5	1210.	0.048
3×10^{-6}	96.1	3572.	0.027	64.9	3909.	0.017
	109.	3374.	0.032	68.3	3774.	0.018
		DIGOXIN			DIGITOXIN	
1×10^{-8}	2.26	8.36	0.270	2.48	3.56	0.697
	1.88	8.36	0.225	2.14	3.66	0.585
3×10^{-8}	5.56	27.3	0.204	-	-	-
	5.59	27.2	0.206	7.33	12.2	0.601
1×10^{-7}	17.1	87.6	0.195	20.4	44.2	0.462
	17.0	90.7	0.187	23.0	48.5	0.474
3×10^{-7}	42.3	302.	0.140	42.0	177.	0.237
	40.5	312.	0.130	42.9	174.	0.246
1×10^{-6}	64.3	1038.	0.062	163.	1271.	0.128
	62.3	1024.	0.061	276.	1379.	0.200
3×10^{-6}	68.2	3311.	0.020	311.	4628.	0.06
	59.4	3379.	0.018	275.	4597.	0.062
		OUABAIN				
1×10^{-8}	1.77	10.3	0.172			
	1.84	9.95	0.185			
3×10^{-8}	5.77	31.5	0.183			
	5.20	31.8	0.164			
1×10^{-7}	15.7	91.5	0.172			
	14.4	95.3	0.151			
3×10^{-7}	35.0	311.	0.112			
	34.9	309.	0.113			
1×10^{-6}	62.7	976.	0.064			
	79.2	1001.	0.079			
3×10^{-6}	120.	3199	0.038			
	93.4	3552.	0.026			

TABLE 24. Experimental binding data for guinea pig heart Na^+K^+-ATPase in the absence of K^+

Glycoside Molarity	bound	unbound	b/u	bound	unbound	b/u
		CONVALLATOXOL			CYMAROL	
1×10^{-8}	5.72	7.24	0.790	5.99	6.16	0.972
	5.48	7.68	0.714	6.24	6.52	0.957
3×10^{-8}	15.2	25.7	0.591	15.7	22.0	0.714
	14.9	26.2	0.569	14.7	20.6	0.714
1×10^{-7}	29.2	78.4	0.372	31.4	86.0	0.365
	28.9	82.0	0.352	32.0	88.0	0.364
3×10^{-7}	43.4	289.	0.150	42.4	308.	0.138
	43.8	293.	0.149	40.6	301.	0.135
		DIGOXIN			DIGITOXIN	
1×10^{-8}	2.91	7.12	0.409	3.84	3.42	1.12
	2.88	7.24	0.398	3.71	3.27	1.13
3×10^{-8}	7.58	23.7	0.320	10.7	13.0	0.823
	7.66	23.5	0.326	11.1	13.4	0.828
1×10^{-7}	19.0	81.6	0.233	22.8	46.4	0.491
	18.8	86.0	0.219	25.0	47.2	0.530
3×10^{-7}	28.8	275.	0.105	36.1	184.	0.196
	30.7	278.	0.110	36.6	182.	0.201
		OUABAIN				
1×10^{-8}	2.99	8.04	0.372			
	2.98	7.84	0.380			
3×10^{-8}	8.22	26.1	0.315			
	8.68	26.3	0.330			
1×10^{-7}	19.4	78.0	0.249			
	20.7	82.0	0.252			
3×10^{-7}	38.7	272.	0.142			
	38.7	280.	0.138			
1×10^{-6}	53.7	876.	0.061			
	53.6	872.	0.061			
3×10^{-6}	67.7	2872.	0.024			
	55.6	2880.	0.019			
1×10^{-5}	80.7	8336.	0.010			
	84.5	9392.	0.009			
3×10^{-5}	121.	26944.	0.004			
	126.	28232.	0.004			

TABLE 25. Experimental binding data for guinea pig heart Na^+K^+-ATPase in the absence of K^+

Glycoside Molarity	bound[a]	unbound[b]	b/u	bound	unbound	b/u
	CONVALLATOXOL			CYMAROL		
1×10^{-8}	5.68	8.72	0.651	5.22	7.68	0.680
	4.63	8.80	0.526	4.84	7.60	0.637
3×10^{-8}	13.7	29.3	0.468	13.0	26.2	0.496
	14.0	29.9	0.468	12.8	25.5	0.502
1×10^{-7}	27.7	83.6	0.331	30.3	106.	0.286
	27.8	93.2	0.298	27.8	97.2	0.286
3×10^{-7}	43.4	315.	0.138	35.6	348.	0.102
	42.2	318.	0.133	31.8	359.	0.088
	DIGOXIN			DIGITOXIN		
1×10^{-8}	2.34	8.52	0.275	3.42	4.20	0.814
	2.06	7.72	0.267	3.40	4.76	0.714
3×10^{-8}	5.57	27.6	0.202	8.68	15.3	0.567
	5.41	28.0	0.193	8.94	16.4	0.545
1×10^{-7}	14.1	90.4	0.166	27.8	47.6	0.584
	17.1	97.2	0.176	17.2	57.6	0.299
3×10^{-7}	25.4	315.	0.081	-	-	-
	33.8	311.	0.109	29.9	212.	0.141
	OUABAIN					
1×10^{-8}	2.36	8.76	0.269			
	2.17	9.28	0.234			
3×10^{-8}	6.67	29.8	0.224			
	6.49	29.5	0.220			
1×10^{-7}	16.2	87.6	0.185			
	17.2	95.6	0.180			
3×10^{-7}	31.2	300.	0.104			
	28.2	307	0.092			

[a] pmoles bound/mg protein.

[b] pM.

TABLE 26. Experimental binding data for guinea pig heart Na^+K^+-ATPase in the absence of K^+

Glycoside Molarity	bound	unbound	b/u	bound	unbound	b/u
		CONVALLATOXOL			CYMAROL	
1×10^{-8}	4.5	8.88	0.507	4.3	8.24	0.522
	4.6	10.6	0.434	-	-	-
3×10^{-8}	10.9	30.0	0.363	10.7	25.8	0.415
	11.5	29.5	0.390	10.7	26.5	0.404
1×10^{-7}	23.5	88.0	0.267	22.0	95.6	0.230
	23.1	96.4	0.240	22.3	100.	0.223
3×10^{-7}	35.0	314.	0.111	30.8	336.	0.092
	33.4	314.	0.106	35.0	327.	0.107
		DIGOXIN			DIGITOXIN	
1×10^{-8}	2.0	8.84	0.226	7.1	4.44	1.60
	1.8	8.56	0.210	6.4	4.76	1.34
3×10^{-8}	5.2	28.1	0.185	-	-	-
	5.1	28.0	0.182	-	-	-
1×10^{-7}	12.4	91.6	0.135	22.3	56.0	0.398
	15.7	96.0	0.164	19.5	66.4	0.294
3×10^{-7}	21.5	314.	0.068	29.6	210.	0.141
	32.3	300.	0.108	27.8	205.	0.136
		OUABAIN				
1×10^{-8}	2.2	9.64	0.228			
	1.9	9.52	0.200			
3×10^{-8}	10.0	52.8	0.189			
	9.6	53.2	0.180			
1×10^{-7}	13.5	91.2	0.148			
	13.6	94.0	0.145			
3×10^{-7}	24.8	319.	0.078			
	28.7	324.	0.088			

TABLE 27. Experimental binding data for guinea pig heart Na^+K^+-ATPase in the absence of K^+

Glycoside Molarity	bound	unbound	b/u	bound	unbound	b/u
		CONVALLATOXOL			CYMAROL	
1×10^{-8}	3.6	7.20	0.500	3.6	6.36	0.566
	3.9	7.20	0.542	3.6	6.12	0.588
3×10^{-8}	8.0	19.8	0.404	9.4	21.0	0.448
	8.7	23.7	0.367	9.0	20.9	0.431
1×10^{-7}	20.6	70.8	0.291	19.8	68.8	0.288
	16.7	74.8	0.223	20.1	79.2	0.254
3×10^{-7}	30.7	253.	0.121	29.4	263.	0.112
	33.7	260.	0.130	41.5	274.	0.151
		DIGOXIN			DIGITOXIN	
1×10^{-8}	1.6	6.60	0.242	2.6	3.42	0.760
	1.6	6.72	0.238	2.5	3.60	0.694
3×10^{-8}	4.4	22.3	0.197	6.7	11.6	0.578
	4.6	23.2	0.198	6.5	11.9	0.546
1×10^{-7}	11.7	74.0	0.158	14.7	47.2	0.311
	11.5	79.6	0.144	16.8	46.8	0.359
3×10^{-7}	18.0	256.	0.070	-	-	-
	18.6	272.	0.068	26.2	158.	0.166
		OUABAIN				
1×10^{-8}	1.8	7.60	0.237			
	2.0	7.60	0.263			
3×10^{-8}	5.5	24.1	0.228			
	4.3	24.6	0.175			
1×10^{-7}	12.9	74.8	0.172			
	13.8	77.2	0.179			
3×10^{-7}	24.6	239.	0.103			
	25.8	246.	0.105			

TABLE 28. Experimental binding data for guinea pig heart Na^+K^+-ATPase in the absence of K^+

Glycoside Molarity	bound[a]	unbound[b]	b/u	bound	unbound	b/u
	CONVALLATOXOL			CYMAROL		
1×10^{-8}	4.64	7.52	0.617	4.36	6.68	0.653
	4.50	7.96	0.565	4.40	6.84	0.643
3×10^{-8}	11.2	24.8	0.452	11.7	22.4	0.522
	11.1	26.4	0.420	12.0	23.1	0.519
1×10^{-7}	24.1	79.6	0.303	24.8	82.4	0.301
	24.0	81.6	0.294	25.4	87.2	0.291
3×10^{-7}	36.1	266.	0.136	34.3	287.	0.120
	47.3	266.	0.178	36.6	287.	0.128
	DIGOXIN			DIGITOXIN		
1×10^{-8}	2.03	7.68	0.264	3.40	3.53	0.963
	1.99	7.72	0.258	3.31	3.74	0.885
3×10^{-8}	5.23	23.8	0.220	8.17	12.8	0.638
	5.07	24.0	0.211	8.74	13.0	0.672
1×10^{-7}	13.1	77.2	0.170	20.0	45.2	0.442
	14.1	82.0	0.172	19.1	47.2	0.405
3×10^{-7}	22.5	270.	0.083	31.8	176.	0.181
	25.0	268.	0.093	31.2	184.	0.170
	OUABAIN					
1×10^{-8}	2.43	8.20	0.296			
	2.25	8.24	0.273			
3×10^{-8}	6.05	26.7	0.226			
	6.59	26.4	0.250			
1×10^{-7}	15.9	80.4	0.198			
	16.4	82.0	0.200			
3×10^{-7}	27.8	273.	0.102			
	31.3	276.	0.113			

TABLE 29. Experimental binding data for guinea pig heart Na^+K^+-ATPase in the presence of 0.625 mM K^+

Glycoside Molarity	bound	unbound	b/u	bound	unbound	b/u
	CONVALLATOXOL			CYMAROL		
1×10^{-8}	3.26	7.88	0.414	3.77	7.88	0.478
	4.22	9.80	0.431	4.99	7.00	0.713
3×10^{-8}	11.9	28.0	0.425	8.00	27.8	0.288
	9.63	29.1	0.331	8.71	27.8	0.313
1×10^{-7}	18.9	81.2	0.233	44.4	87.6	0.507
	20.3	95.2	0.213	45.3	94.0	0.482
3×10^{-7}	57.3	336.	0.170	57.8	327.	0.177
	63.2	337.	0.188	58.5	326.	0.179
	DIGOXIN			DIGITOXIN		
1×10^{-8}	2.38	9.80	0.243	-	-	-
	2.37	10.1	0.235	2.65	4.24	0.625
3×10^{-8}	5.84	30.4	0.192	5.88	13.8	0.498
	5.64	30.6	0.184	7.62	13.0	0.586
1×10^{-7}	14.9	92.4	0.161	35.6	55.6	0.640
	16.2	96.4	0.168	42.0	58.0	0.724
3×10^{-7}	47.8	321.	0.149	59.5	187.	0.318
	44.9	310	0.145	60.6	197.	0.308
	OUABAIN					
1×10^{-8}	1.97	10.4	0.189			
	1.85	10.3	0.180			
3×10^{-8}	5.46	31.3	0.174			
	5.36	32.0	0.168			
1×10^{-7}	12.0	99.2	0.121			
	12.7	110.	0.115			
3×10^{-7}	46.0	312.	0.147			
	46.6	314.	0.148			

TABLE 30. Experimental data for the binding of convallatoxol to guinea pig kidney Na^+K^+-ATPase in the presence of ATP or inorganic phosphate

Convallatoxol Molarity	bound	unbound	b/u	bound	unbound	b/u
	ATP			H_3PO_4		
1×10^{-8}	6.62	1.19	5.56	5.65	0.905	6.24
	6.43	1.13	5.69	5.65	0.946	5.97
3×10^{-8}	19.6	3.96	4.95	19.2	3.78	5.08
	18.9	3.98	4.75	18.4	3.95	4.66
1×10^{-7}	49.3	17.8	2.77	40.6	16.4	2.48
	50.8	19.0	2.67	41.5	16.0	2.59
3×10^{-7}	90.7	83.6	1.08	79.2	85.9	0.922
	96.8	84.8	1.14	79.9	86.9	0.919
1×10^{-6}	115.	319.	0.360	97.6	283.	0.345
	110.	310.	0.355	95.0	266.	0.357
3×10^{-6}	112.	1102.	0.102	106.	1068.	0.099
	125.	1097.	0.114	108.	1101.	0.098
1×10^{-5}	131.	2278.	0.058	118.	2623.	0.045
	185.	2291.	0.081	159.	2700.	0.059
3×10^{-5}	200.	8834.	0.023	186.	8576.	0.022
	252.	8726.	0.029	164.	8532.	0.019

TABLE 31. Rat Brain ATPase Activity as a Function of Time

Time in Minutes	A_{660}	Total Activity	A_{660}	Mg^{2+} [a] Activity
Study 1[b]				
2.5	.090	12.0	.038	5.0
	.081	10.8	.035	4.6
5.0	.180	24.0	.058	7.7
	.180	24.0	.052	6.9
10.0	.342	45.5	.078	10.4
	.365	48.6	.068	9.0
15.0	.50	66.6	.110	14.6
	.50	66.6	.109	14.5
20.0	.62	82.5	.121	16.1
	.62	82.5	.138	18.4
30.0	.80	106.5	.142	18.9
	.96	127.8	.142	18.9
Study 2[c]				
2.5	.120	14.6	.025	3.0
	.112	13.6	.025	3.0
5.0	.210	25.5	.039	4.7
	.213	25.9	.041	5.0
10.0	.380	46.2	.061	7.4
	.359	43.6	.063	7.7
15.0	.47	57.1	.070	8.5
	.47	57.1	.070	8.5
20.0	.61	74.2	.110	13.4
	.57	69.3	.110	13.4
30.0	.75	91.2	.139	16.9
	.75	91.2	.142	17.3

[a] inhibited by 1×10^{-2} M Ouabain.
[b] 1.16 A_{660} units/μmole P_i.
[c] 1.27 A_{660} units/μmole P_i.

TABLE 32. Rat ATPase Activities as Functions of Enzyme Concentration

BRAIN			HEART		
µl Enzyme solution [a]	A_{660}	Activity [b]	µl Enzyme solution [c]	A_{660}	Activity [d]
10	.080	11.6	Study 1		
	.070	10.1			
25	.158	22.8	10	.079	4.7
	.165	23.8		-	-
50	.290	41.9	25	.137	8.2
	.302	43.6		.152	9.0
75	.442	63.8	50	.291	17.3
	.441	63.7		.289	17.2
	.54	78.0	75	.427	25.4
	.55	79.4		.417	24.8
			100	.517	30.8
				.497	29.6
			Study 2		
			10	.118	4.6
				.113	4.4
			25	.242	9.5
				.244	9.5
			50	.46	18.0
				.47	18.4
				.48	18.6
				.45	17.6
			75	.69	27.0
				.66	25.8
			100	.84	32.8
				.86	33.6

[a] 0.045 mg Pro/50 µl

[b] 1.20 A_{660} units/µmole P_i

[c] 0.165 mg Pro/50 µl

[d] 1.31 A_{660} units/µmole P_i

TABLE 33. Rat ATPase Activities as Functions of KCl Concentration

KCl mM	BRAIN				HEART	
	A_{660}	Total[a] Activity	A_{660}	Mg^{2+}[b] Activity	A_{660}	Total[c] Activity
0.155	.132	17.7	.095	12.8	.240	15.0
	.131	17.6	.089	11.9	.240	15.0
	.140	18.8			.232	14.5
0.310	.161	21.6	.093	12.5	.270	16.8
	.165	22.1	.087	11.7	.269	16.8
	-	-			.260	16.2
0.625	.231	31.0	.090	12.1	.310	19.3
	.230	30.9	.090	12.1	.310	19.3
	.228	30.6			.302	18.8
1.25	.211	28.6	.093	12.5	.382	23.8
	.221	30.0	.090	12.1	.380	23.7
	.210	28.4			.344	21.5
2.50	.320	43.0	.100	13.4	.380	23.7
	.327	43.9	.096	12.9	.376	23.5
	.324	43.5			.365	22.8
5.00	.280	37.6	-	-	.340	21.2
	.300	40.3	.100	13.4	.358	22.3
	.280	37.6			.350	21.8
10.00	.235	31.5	.102	13.7	.330	20.6
	.239	32.1	.099	13.3	.329	20.5
	.239	32.1			.336	21.0
20.00	.208	27.9	.093	12.5	.312	19.5
	.209	28.0	.091	12.2	.320	20.0
	.202	27.1			.335	20.9

[a] 1.20 A_{660} units/μmole P_i.

[b] inhibited with 5×10^{-4} M Ouabain.

[c] 1.25 A_{660} units/μmole P_i.

TABLE 34. Inhibition of Rat Brain ATPase by Various Gardiac Glycosides at Several K^+ Concentrations

Glycoside Molarity	CONVALLATOXOL A_{660}	CONVALLATOXOL % Inhibition	DIGOXIN A_{660}	DIGOXIN % Inhibition	CYMAROL A_{660}	CYMAROL % Inhibition
2.5 mM K^+ [a]						
1×10^{-8}	.420	- 10	.470	- 30	-	-
	.415	- 8	.500	- 42	.435	- 16
5×10^{-8}	.332	25	.420	- 10	.370	10
	.342	21	.450	- 22	.385	4
1×10^{-7}	-	-	.440	- 18	.357	15
	.375	8	.445	- 20	.367	11
5×10^{-7}	.288	43	.340	22	.280	46
	.302	37	.330	26	.278	47
1×10^{-6}	.272	49	.310	34	.265	52
	.302	37	.315	32	.257	55
5×10^{-6}	.250	58	.239	63	.221	70
	.255	56	.249	59	.245	60
1×10^{-5}	.245	60	.221	70	.209	75
	.278	47	.230	66	.215	72
5×10^{-5}	.200	78	.121	110	.140	102
	.220	70	.130	106	.145	100
0.625 mM K^+ [b]						
1×10^{-8}	.235	- 11	.265	- 40	.247	- 22
	.254	- 29	.256	- 31	.250	- 25
5×10^{-8}	.174	48	.235	- 11	.250	- 25
	.199	24	.255	- 30	.222	2
1×10^{-7}	.214	10	.235	- 11	.220	4
	.206	17	.245	- 20	.230	6
5×10^{-7}	.183	40	.186	37	.182	40
	.185	38	.193	30	.190	33
1×10^{-6}	.174	48	.175	47	.170	52
	.185	38	-	-	.199	24
5×10^{-6}	.165	57	.156	66	.155	66
	.155	66	.160	62	.175	47
1×10^{-5}	.153	68	.154	67	.161	61
	.164	58	-	-	.168	54
5×10^{-5}	.125	95	.105	115	.145	76
	.126	94	.135	86	.130	91

[a] 1.16 A_{660} units/µmole P_i; Na^+K^+-ATPase S.A. - .395 A_{660} in absence of glycoside.

[b] 1.16 A_{660} units/µmole P_i; Na^+K^+-ATPase S.A. - .224 A_{660} in absence of glycoside.

TABLE 34 (continued)

Glycoside Molarity	OUABAIN A_{660}	OUABAIN % Inhibition	DIGITOXIN A_{660}	DIGITOXIN % Inhibition	HELLEBRIN A_{660}	HELLEBRIN % Inhibition
2.5 mM K^+						
1×10^{-8}	.451	- 22	.490	- 38	.388	3
	.493	- 39	.470	- 30	.389	3
5×10^{-8}	.409	- 5	.389	2	.304	37
	.419	- 10	.390	2	.312	33
1×10^{-7}	.385	4	.408	- 5	.271	50
	.425	- 12	.380	6	.289	43
5×10^{-7}	.297	39	.280	46	.210	74
	.303	37	.283	45	.226	68
1×10^{-6}	.290	42	.280	46	.218	71
	.325	28	.289	43	.221	70
5×10^{-6}	.249	59	.238	63	<.170	>90
	.272	49	.220	70	<.170	>90
1×10^{-5}	.227	67	.228	67	.170	90
	.233	65	.235	64	.181	86
5×10^{-5}	.187	84	.160	94	<.170	>90
	.220	70	.160	94	<.170	>90
1×10^{-4}	.153	97				
	.161	94				
5×10^{-4}	.135					
	.135					
1×10^{-2}	.135	100				
	.135					
0.625 mM K^+						
1×10^{-8}	.300	- 73	.218	6	.221	3
	.273	- 47	.221	3	.246	21
5×10^{-8}	.240	- 16	.192	31	.197	26
	.239	- 14	.202	20	.191	32
1×10^{-7}	.233	- 9	.203	20	.180	42
	.254	- 29	.208	15	.176	46
5×10^{-7}	.201	22	.170	52	.145	76
	.210	13	.170	52	.158	64
1×10^{-6}	.199	24	.172	50	.140	81
	.210	13	.179	43	.167	55
5×10^{-6}	.190	33	.146	75	.105	115
	.181	41	.145	76	.115	105
1×10^{-5}	.188	35	.159	63	.132	89
	-	-	.162	60	.135	86
5×10^{-5}	.159	63	.120	100	.097	122
	.186	37	.136	85	.103	117
1×10^{-4}	.148	73				
	.148	73				
5×10^{-4}	.120					
	.122					
1×10^{-2}	.118	100				
	.120					

TABLE 34 (continued)

Glycoside Molarity	CONVALLATOXOL A_{660}	CONVALLATOXOL % Inhibition	DIGOXIN A_{660}	DIGOXIN % Inhibition	CYMAROL A_{660}	CYMAROL % Inhibition
20.0 mM K^+[c]						
1×10^{-8}	.308	- 7	.291	3	.328	- 19
	.307	- 6	.318	- 13	.326	- 18
5×10^{-8}	.297	0	.301	- 3	.308	- 7
	.291	3	.296	0	.300	- 2
1×10^{-7}	.272	15	.286	6	.276	12
	.279	10	.288	5	.276	12
5×10^{-7}	.244	32	-	-	.247	30
	.249	28	-	-	.247	30
1×10^{-6}	.217	48	.258	23	.227	42
	.219	46	.258	29	.218	47
5×10^{-6}	.165	79	.198	59	.164	80
	.177	72	.206	54	.167	78
1×10^{-5}	.155	85	.191	63	.157	84
	.147	90	.188	65	.150	88
5×10^{-5}	.137	96	.194	62	.139	95
	.139	95	.202	57	.139	95
1×10^{-4}	.132	99	.155	85	.138	95
	.128	101	.161	81	.136	96
5×10^{-4}	.122	105	.078	131	.126	102
	.129	101	.046	151	.123	104
0.625 mM K^+[d]						
1×10^{-8}	.142	21	-	-	.163	- 7
	.153	6	.169	- 16	.139	25
5×10^{-8}	.140	24	.146	16	.112	62
	.142	21	.154	5	.128	40
1×10^{-7}	.122	48	.143	20	.118	54
	.141	23	.143	20	.116	57
5×10^{-7}	.128	40	.124	46	.108	68
	.129	39	-	-	.127	42
1×10^{-6}	.115	58	.121	50	.103	74
	.112	62	.125	44	.109	66
5×10^{-6}	.118	54	.115	58	.097	83
	.114	59	.115	58	.098	81
1×10^{-5}	.113	61	.098	81	.088	95
	.113	61	.100	79	.097	83
5×10^{-5}	.101	77	.077	110	.085	99
	.103	74	.095	85	.087	96

[c] 1.20 A_{660} units/μmole P_i; Na^+K^+-ATPase S.A. - .296 A_{660} in absence of glycoside.

[d] 1.27 A_{660} units/μmole P_i; Na^+K^+-ATPase S.A. - .213 A_{660} in absence of glycoside.

TABLE 34 (continued

Glycoside Molarity	OUABAIN A_{660}	OUABAIN Inhibition	DIGITOXIN A_{660}	DIGITOXIN Inhibition	HELLEBRIN A_{660}	HELLEBRIN Inhibition
20.0 mM K^+						
1×10^{-8}	.318	- 13	.317	- 12		
	.318	- 13	.306	- 6		
5×10^{-8}	.298	- 1	.298	- 1		
	.270	16	.299	- 2		
1×10^{-7}	.279	10	.291	3	.298	- 1
	.288	5	.296	1	.288	5
5×10^{-7}	.260	22	.236	36	.234	38
	.260	22	.246	30	.235	37
1×10^{-6}	.253	26	-	-	.226	42
	.250	28	.224	44	.233	38
5×10^{-6}	.196	60	.176	72	.183	68
	.188	65	.178	71	.178	71
1×10^{-5}	.168	77	.164	80	.169	77
	.183	68	.166	78	.169	77
5×10^{-5}	.140	94	.180	70	.146	90
	.143	92	.188	65	.143	92
1×10^{-4}	.156	84	.141	94		
	.146	90	.153	86		
5×10^{-4}	.133	100	.082	129		
	.128		.083	128		
0.625 mM K^+						
1×10^{-8}	.170	- 17	.170	- 17	.151	9
	.156	2	.170	- 17	.148	13
5×10^{-8}	.154	5	.160	- 3	.136	29
	.149	12	.162	- 6	.138	27
1×10^{-7}	.141	23	.164	- 9	.128	40
	.141	23	.151	9	.135	31
5×10^{-7}	.122	48	.135	31	.121	50
	.132	35	.139	25	.116	57
1×10^{-6}	-	-	.142	21	.126	43
	-	-	.132	35	.126	43
5×10^{-6}	-	-	.122	48	.106	70
	-	-	.125	44	.108	68
1×10^{-5}	.117	55	.111	64	.105	72
	.119	53	.110	65	.107	69
5×10^{-5}	-	-	.090	92	.096	84
	.115	58	.088	95	.096	84
1×10^{-4}	.107	69				
	.108	68				
5×10^{-4}	.088	95				
	.092	90				

TABLE 35. Experimental binding data for rat brain Na^+K^+-ATPase in the absence of K^+

Glycoside Molarity	bound	unbound	b/u	bound	unbound	b/u
	CONVALLATOXOL			CYMAROL		
1×10^{-8}	23.4	4.91	4.76	15.5	5.45	2.84
	21.5	5.02	4.28	16.1	5.01	3.21
3×10^{-8}	50.6	22.6	2.24	35.3	19.0	1.86
	50.6	22.0	2.30	35.5	20.1	1.77
1×10^{-7}	63.3	92.8	0.68	41.8	82.9	0.50
	60.2	90.7	0.66	40.5	89.3	0.45
3×10^{-7}	65.1	305.	0.21	44.6	304.	0.15
	58.3	322.	0.18	45.4	309.	0.15
	DIGOXIN			DIGITOXIN		
1×10^{-8}	7.47	8.24	0.91	11.0	2.60	4.23
	6.92	8.48	0.82	10.8	2.33	4.64
3×10^{-8}	21.7	28.2	0.77	32.4	10.1	3.21
	20.2	29.2	0.69	32.1	11.6	2.77
1×10^{-7}	34.3	83.7	0.41	54.0	48.7	1.11
	44.8	92.1	0.49	70.3	44.0	1.60
3×10^{-7}	36.5	299.	0.12	70.0	200.	0.35
	39.4	318.	0.12	53.9	177.	0.30
	OUABAIN					
1×10^{-8}	12.8	10.2	1.25			
	12.9	9.46	1.36			
3×10^{-8}	32.1	32.4	0.99			
	26.4	28.6	0.92			
1×10^{-7}	51.9	97.1	0.53			
	49.3	95.2	0.52			
3×10^{-7}	57.3	338.	0.17			
	52.4	340.	0.15			

TABLE 36. Experimental binding data for rat brain Na^+K^+-ATPase in the absence of K^+

Glycoside Molarity	bound	unbound	b/u	bound	unbound	b/u
	CONVALLATOXOL			CYMAROL		
1×10^{-8}	18.0	2.67	6.74	11.7	2.02	5.79
	18.2	2.42	7.52	11.8	2.05	5.76
3×10^{-8}	54.2	11.3	4.80	34.3	8.26	4.15
	55.0	10.7	5.14	35.6	7.53	4.73
1×10^{-7}	104.	81.1	1.28	84.9	35.9	2.36
	102.	83.9	1.22	90.8	39.6	2.29
3×10^{-7}	121.	458.	0.264	123.	269.	0.457
	118	460.	0.256	131.	268.	0.489
	DIGOXIN			DIGITOXIN		
1×10^{-8}	10.5	6.23	1.68	7.68	1.59	4.83
	10.2	7.11	1.43	7.99	1.72	4.64
3×10^{-8}	30.3	25.0	1.21	26.0	5.38	4.83
	31.0	22.8	1.36	27.0	5.38	5.02
1×10^{-7}	77.7	69.0	1.13	-	23.0	-
	72.4	69.7	1.04	80.8	25.8	3.13
3×10^{-7}	125.	320.	0.391	211.	122.	1.73
	127.	328.	0.387	205.	123.	1.67
	OUABAIN					
1×10^{-8}	11.0	6.53	1.68			
	11.2	6.78	1.65			
3×10^{-8}	-	-	-			
	-	-	-			
1×10^{-7}	104.	123.	0.846			
	99.2	133.	0.746			
3×10^{-7}	118.	380.	0.310			
	108.	405.	0.267			

TABLE 37. Experimental binding data for rat brain Na^+K^+-ATPase in the presence of 0.625 mM K^+

Glycoside Molarity	bound	unbound	b/u	bound	unbound	b/u
	CONVALLATOXOL			CYMAROL		
1×10^{-8}	21.1	5.91	3.57	10.8	6.44	1.68
	18.8	6.11	3.08	12.8	6.38	2.01
3×10^{-8}	39.7	24.6	1.61	27.1	22.6	1.20
	39.4	24.3	1.62	28.6	23.0	1.24
1×10^{-7}	54.4	85.8	0.634	39.7	86.1	0.461
	25.9	88.3	0.293	40.1	90.8	0.442
3×10^{-7}	52.6	323.	0.163	45.7	318.	0.144
	53.9	321.	0.167	37.5	311.	0.120
	DIGOXIN			DIGITOXIN		
1×10^{-8}	5.08	9.22	0.551	10.4	3.16	3.29
	6.14	9.15	0.671	11.3	3.18	3.55
3×10^{-8}	14.8	29.9	0.495	28.9	11.7	2.47
	13.5	31.5	0.428	30.4	11.9	2.55
1×10^{-7}	24.0	95.0	0.253	56.6	46.1	1.23
	27.8	93.1	0.299	83.0	47.6	1.74
3×10^{-7}	63.2	319.	0.198	97.8	180.	0.543
	46.1	325.	0.142	161.	151.	1.07
	OUABAIN					
1×10^{-8}	7.58	11.6	0.653			
	8.06	11.2	0.720			
3×10^{-8}	18.7	37.4	0.500			
	20.4	37.0	0.551			
1×10^{-7}	40.3	105.	0.384			
	34.6	104.	0.333			
3×10^{-7}	41.0	352.	0.116			
	43.6	360.	0.121			

TABLE 38. Experimental data for rat brain Na^+K^+-ATPase/1 x 10^{-8} M convallatoxol binding in the presence of various K^+ concentrations

KCl mM	bound	unbound	b/u
0	39.6	5.39	7.35
	38.4	5.27	7.29
	39.1	5.06	7.73
0.155	37.5	5.47	6.86
	36.1	5.95	6.07
	39.2	5.40	7.26
0.625	29.9	7.30	4.10
	31.0	7.08	4.38
	30.1	6.34	4.75
2.5	16.3	11.1	1.47
	15.6	11.4	1.37
	14.9	11.3	1.32

TABLE 39. Experimental data for rat brain Na^+K^+-ATPase/convallatoxol binding in the presence of various concentrations of K^+

Glycoside Molarity	bound	unbound	b/u	bound	unbound	b/u
		0.155 mM K^+			0.625 mM K^+	
1×10^{-8}	32.4	2.99	10.8	23.4	4.36	5.37
	33.7	2.96	11.4	26.7	4.10	6.51
	53.0	1.55	34.2	34.5	4.52	7.63
	50.4	1.61	31.3	34.1	3.96	8.61
3×10^{-8}	80.8	14.0	5.77	70.5	16.6	4.25
	79.7	13.8	5.78	64.8	16.0	4.05
	97.9	16.8	5.83	75.7	20.8	3.64
	98.0	17.5	5.60	74.6	22.0	3.39
		1.25 mM K^+			2.5 mM K^+	
1×10^{-8}	31.3	2.58	12.1	12.7	7.02	1.81
	31.7	2.73	11.6	13.3	6.80	1.96
	37.0	3.06	12.1	16.6	8.74	1.90
	36.3	3.15	11.5	14.4	8.56	1.68
3×10^{-8}	68.4	14.1	4.85	33.6	23.4	1.44
	69.2	14.4	4.80	30.8	23.1	1.33
	86.8	18.6	4.67	-	-	-
	89.8	17.8	5.04	44.2	29.9	1.48
		5.0 mM K^+			20.0 mM K^+	
1×10^{-8}	8.26	8.58	0.96	2.42	10.1	0.24
	8.23	9.03	0.91	2.60	10.1	0.26
	11.4	10.6	1.08	-	-	-
	9.82	10.7	0.92	3.04	11.9	0.25
3×10^{-8}	21.0	26.9	0.79	7.34	30.5	0.24
	20.0	27.6	0.72	6.82	30.9	0.22
	22.8	34.5	0.66	7.72	39.0	0.20
	25.2	35.4	0.71	8.36	39.6	0.21
		Without K^+				
1×10^{-8}	32.8	2.34	14.0			
	31.8	2.51	12.7			
3×10^{-8}	76.5	13.5	5.67			
	77.6	13.1	5.92			

TABLE 40. Experimental binding data for the rate of association and dissociation of convallatoxol to rat brain Na^+K^+-ATPase

Time in Minutes	bound	unbound	b/u	bound	unbound	b/u
ASSOCIATION						
2.5	17.3	11.2	1.54			
	18.4	11.0	1.67			
5.0	23.3	10.0	2.33			
	24.4	10.2	2.39			
10.0	25.2	9.81	2.57			
	25.5	10.0	2.55			
15.0	25.7	10.4	2.47			
	25.9	10.3	2.51			
20.0	25.5	10.1	2.52			
	25.7	9.98	2.58			
30.0	24.5	10.3	2.38			
	22.2	10.0	2.22			
45.0	22.6	10.4	2.17			
	22.0	11.4	1.93			
60.0	21.6	11.1	1.95			
	21.0	10.9	1.93			
DISSOCIATION						
2.5	16.0	10.4	1.54	14.6	1.43	10.2
	13.9	10.6	1.31	12.6	1.30	9.69
5.0	13.4	10.8	1.24	11.8	1.64	7.20
	13.7	10.4	1.32	12.0	1.68	7.14
10.0	10.4	11.0	0.944	8.26	2.12	3.90
	11.5	10.7	1.07	9.22	2.27	4.06
15.0	11.6	10.0	1.16	8.97	2.64	3.40
	9.84	10.9	0.903	7.58	2.26	3.35
20.0	8.10	10.5	0.771	5.22	2.88	1.81
	8.26	10.9	0.758	5.72	2.54	2.25
30.0	7.18	10.6	0.677	3.98	3.20	1.24
	-	-	-	-	-	-
45.0	7.52	10.5	0.716	3.95	3.57	1.11
	7.20	10.4	0.692	3.89	3.31	1.18
60.0	6.08	10.7	0.568	2.48	3.60	0.689
	7.56	10.9	0.694	3.64	3.92	0.928

TABLE 41. Experimental binding data for the rate of association and dissociation of digoxin to rat brain Na^+K^+-ATPase

Time in Minutes	bound	unbound	b/u	bound	unbound	b/u
ASSOCIATION						
2.5	8.40	10.7	0.785			
	7.88	11.0	0.716			
5.0	11.4	10.1	1.13			
	11.1	9.81	1.13			
10.0	18.9	8.05	2.35			
	19.1	7.98	2.39			
15.0	21.6	8.09	2.67			
	19.9	7.84	2.54			
20.0	23.4	7.41	3.16			
	23.6	7.90	2.99			
30.0	26.4	6.99	3.78			
	27.4	7.01	3.91			
45.0	28.4	6.70	4.24			
	27.1	6.14	4.41			
60.0	28.9	5.99	4.82			
	28.4	5.81	4.89			
DISSOCIATION						
2.5	8.40	10.2	0.823	7.65	0.747	10.2
	-	10.2	-	-	-	-
5.0	8.17	11.5	0.710	7.37	0.796	9.26
	9.01	10.6	0.850	8.08	0.928	8.71
10.0	8.05	11.3	0.712	7.11	0.940	7.56
	7.73	10.5	0.736	6.73	1.00	6.73
16.0	7.54	10.8	0.698	6.34	1.20	5.28
	7.27	11.2	0.649	6.24	1.03	6.06
20.0	7.19	11.0	0.654	6.18	1.01	6.12
	6.97	11.6	0.601	5.86	1.11	5.28
30.0	7.96	11.4	0.698	6.58	1.38	4.77
	6.50	10.9	0.596	5.40	1.10	4.91
45.0	6.62	10.4	0.636	5.40	1.22	4.43
	5.92	10.7	0.553	4.74	1.18	4.02
60.0	5.75	10.5	0.548	4.36	1.39	3.14
	5.86	10.8	0.542	4.47	1.39	3.22

TABLE 42. Experimental binding data for the rate of association of glycosides to guinea pig heart Na^+K^+-ATPase

Time in Minutes	bound	unbound	b/u	bound	unbound	b/u
CONVALLATOXOL[a]						
2.5	8.52	19.4	0.439			
	7.16	20.2	0.354			
5.0	6.49	20.5	0.316			
	6.64	20.1	0.330			
10.0	5.38	20.4	0.264			
	5.64	21.5	0.262			
20.0	5.87	20.8	0.282			
	5.58	21.8	0.256			
30.0	-	-	-			
	5.04	21.2	0.238			
CONVALLATOXOL[b]						
2.5	4.28	8.60	0.498	2.69	9.20	0.292
	4.22	8.52	0.495	2.59	8.96	0.289
5.0	4.41	8.44	0.522	2.66	9.24	0.288
	5.16	8.20	0.629	2.70	9.28	0.291
15.0	-	-	-	-	-	-
	-	-	-	-	-	-
20.0	-	-	-	2.68	9.16	0.292
	4.45	8.36	0.532	2.71	9.44	0.287
30.0	4.30	8.64	0.498	2.78	9.36	0.297
	4.02	8.24	0.488	2.74	9.48	0.289
OUABAIN[c]						
2.5	4.75	22.6	0.210			
	5.03	23.4	0.215			
5.0	5.45	22.9	0.238			
	5.23	23.2	0.225			
20.0	6.08	22.5	0.270			
	5.43	22.4	0.242			
30.0	4.86	23.0	0.211			
	6.62	24.1	0.275			

[a] 3×10^{-8} M

[b,c] 1×10^{-8} M

TABLE 43. Experimental binding data for the rate of association of convallatoxol and cymarol to guinea pig brain Na^+K^+-ATPase

Time in Minutes	bound	unbound	b/u
	CONVALLATOXOL[a]		
2.5	24.8	5.45	4.55
	23.8	6.66	3.57
5.0	22.9	5.97	3.84
	24.1	5.71	4.22
10.0	25.2	5.21	4.84
	25.4	4.38	5.80
20.0	26.6	3.54	7.51
	24.2	4.47	5.41
30.0	26.1	3.23	8.08
	25.5	3.73	6.84
	CYMAROL[b]		
5.0	26.7	4.20	6.36
	26.7	4.75	5.62
10.0	27.0	4.15	6.51
	27.4	4.04	6.78
20.0	27.4	3.95	6.94
	28.0	3.46	8.09
30.0	27.6	3.44	8.02
	29.0	3.70	7.84

[a] 3×10^{-8} M

[b] 3×10^{-8} M

TABLE 44. Experimental binding data for the rate of association and dissociation of convallatoxol to guinea pig brain Na^+K^+-ATPase

Time in Minutes	bound	unbound	b/u	bound	unbound	b/u
ASSOCIATION						
2.5	14.3	5.75	2.49			
	14.7	6.00	2.45			
5.0	15.7	4.10	3.83			
	16.0	4.34	3.69			
10.0	18.9	2.92	6.47			
	18.7	2.80	6.68			
15.0	18.4	2.47	7.45			
	18.3	2.61	7.01			
20.0	18.4	2.14	8.60			
	18.3	2.28	8.03			
30.0	19.6	1.88	10.4			
	20.0	2.10	9.52			
DISSOCIATION						
2.5	16.7	3.91	4.28	14.6	2.15	6.79
	15.5	3.94	3.93	13.5	1.99	6.78
5.0	15.3	3.79	4.04	12.4	2.92	4.25
	14.9	3.92	3.80	12.1	2.79	4.34
10.0	15.4	3.88	3.96	11.3	4.06	2.78
	14.9	3.88	3.84	10.8	4.10	2.63
15.0	14.3	4.05	3.54	9.46	4.88	1.92
	14.6	4.18	3.50	9.90	4.72	2.10
20.0	13.7	4.10	3.35	8.51	5.21	1.63
	14.2	4.14	3.42	8.85	5.33	1.66
30.0	13.4	3.98	3.35	7.08	6.27	1.13
	11.5	4.22	2.72	7.39	4.13	1.79

TABLE 46. Rat Heart ATPase Activity as a Function of Time

Time in Minutes	A_{660}	Total Activity	A_{660}	Mg^{2+}[a] Activity
Study 1[b]				
2.5	.125	4.3	.026	0.9
	.126	4.3	.028	1.0
5.0	.218	7.5	.050	1.7
	.192	6.6	.043	1.5
10.0	.259	8.9	.050	1.7
	.272	9.3	.049	1.7
15.0	.331	11.4	.090	3.1
	.320	11.0	.096	3.3
20.0	.332	11.4	.100	3.4
	.340	11.7	.109	3.7
30.0	.45	15.4	.150	5.1
	.47	16.1	.150	5.1
Study 2[c]				
2.5	.199	7.8		
	.191	7.5		
5.0	.263	10.3		
	.261	10.2		
10.0	.41	15.9		
	.42	16.5		
15.0	.59	23.1		
	.57	22.3		
20.0	.69	27.0		
	.65	25.4		
30.0	.95	37.2		
	.87	34.0		

[a] inhibited by 1 x 10^{-2} M Ouabain.
[b] 1.27 A_{660} units/μmole P_i.
[c] 1.21 A_{660} units/μmole P_i.

TABLE 47. Inhibition of Rat Heart ATPase by Various Cardiac Glycosides at Two K^+ Concentrations

Glycoside Molarity	CONVALLATOXOL A_{660}	CONVALLATOXOL Inhibition	DIGOXIN A_{660}	DIGOXIN Inhibition	CYMAROL A_{660}	CYMAROL Inhibition
20.0 mM K^+[a]						
1×10^{-7}	.50	1				
	.55	- 23				
5×10^{-7}	.51	- 4				
	.49	5				
1×10^{-6}	.50	- 2	.441		.422	36
	.50	1	.431	31	.452	21
5×10^{-6}	.49	5	.436	29	.427	33
	.49	5	.436	29	.442	26
1×10^{-5}	.45	24	.421	36	.422	36
	.47	15	.451	22	.417	38
5×10^{-5}	.47	15	.406	43	.403	45
	.46	20	.401	46	.406	43
1×10^{-4}	.45	22	.319	84	.414	39
	.49	5	.331	79	.427	33
5×10^{-4}	.43	34	-	-	.367	62
	.42	36	-	-	.387	52
0.625 mM K^+[b]						
1×10^{-7}	.474	3				
	.489	- 6				
5×10^{-7}	.484	- 3				
	.494	- 8				
1×10^{-6}	.484	- 3	.42	32	.404	40
	.494	- 8	.42	32	.444	19
5×10^{-6}	.464	8	.370	59	.399	43
	.464	8	.370	59	.414	35
1×10^{-5}	.444	19	.350	70	.384	51
	.464	8	.350	70	.384	51
5×10^{-5}	.434	24	.265	116	.324	84
	.424	30	.267	115	.334	78
1×10^{-4}	.404	40	-	-	.344	73
	.404	40	-	-	.334	78
5×10^{-4}	.359	65	-	-	.324	84
	.359	65	-	-	.309	92

[a] 1.23 A_{660} units/µmole P_i; Na^+K^+-ATPase 8.1; .50 A_{660} in absence of glycoside.

[b] 1.23 A_{660} units/µmole P_i; Na^+K^+-ATPase 7.1; .48 A_{660} in absence of glycoside.

TABLE 47 (continued)

Glycoside Molarity	OUABAIN A_{660}	OUABAIN % Inhibition	DIGITOXIN A_{660}	DIGITOXIN % Inhibition	HELLEBRIN A_{660}	HELLEBRIN % Inhibition
20.0 mM K^+						
1×10^{-6}	.492	2	.433	30	.428	33
	.527	- 14	.458	18	.438	28
5×10^{-6}	.457	19	.453	21	.430	32
	.442	26	.443	26	.425	34
1×10^{-5}	.436	29	.453	21	.400	46
	.452	21	.458	18	.418	38
5×10^{-5}	.412	40	.468	14	.390	51
	.422	36	.458	18	.380	56
1×10^{-4}	.402	45	.313	87	.380	56
	.402	45	.318	85	.380	56
5×10^{-4}	.352	69	-	-	.340	74
	.342	74	-	-	.360	65
1×10^{-3}	.332	78				
	.347	71				
5×10^{-3}	.302	92				
	.302	92				
1.2×10^{-2}	.282	10.8				
	.292	11.2				
0.625 mM K^+						
1×10^{-6}	.444	19	.449	16	.429	27
	.454	13	.454	13	.444	19
5×10^{-6}	.414	35	.414	35	-	-
	.414	35	.416	34	-	-
1×10^{-5}	.411	37	.384	51	.369	59
	.424	30	.394	46	.379	54
5×10^{-5}	.356	66	.294	100	-	-
	.360	64	.298	98	-	-
1×10^{-4}	.334	78	-	-	.329	81
	.344	73	-	-	.329	81
5×10^{-4}	.319	86	-	-	.299	97
	.324	84	-	-	.309	92
1×10^{-3}	.334	78				
	.314	89				
5×10^{-3}	.294	100				
	.324	84				
1.2×10^{-2}	.294	100				
	.294					

TABLE 48. Experimental binding data for the rates of association of convallatoxol and cymarol to rat heart Na^+K^+-ATPase

Time in Minutes	bound	unbound	b/u
CONVALLATOXOL			
2.5	.552	12.3	.0449
	.400	12.5	.0320
5.0	.373	11.9	.0313
	.304	11.9	.0255
10.0	.759	12.2	.0622
	-	11.7	-
20.0	.262	11.5	.0228
	.414	12.0	.0345
30.0	.304	12.2	.0249
	.304	11.8	.0258
60.0	.276	11.9	.0232
	-	-	-
CYMAROL			
2.5	.165	9.99	.0165
	.195	9.54	.0204
5.0	.165	9.79	.0168
	.270	9.40	.0287
10.0	.088	9.98	.0098
	.098	10.2	.0096
20.0	.112	10.5	.0107
	.135	10.5	.0128
30.0	.075	9.82	.0076
	.098	10.4	.0094
60.0	.112	9.55	.0117
	.045	9.76	.0046

TABLE 49. Experimental data for rat heart Na^+K^+-ATPase/convallatoxol binding in the presence of various K^+ concentrations

Glycoside Molarity	bound	unbound	b/u	bound	unbound	b/u
		0.155 mM K^+			0.625 mM K^+	
1×10^{-8}	.373	12.6	.0296	.290	11.8	.0246
	.235	11.8	.0199	.152	11.9	.0128
3×10^{-8}	.745	38.0	.0196	.538	38.4	.0140
	.800	38.9	.0206	.373	34.8	.0107
1×10^{-7}	.913	111.	.0082	.845	105.	.0080
	.744	109.	.0068	.304	108.	.0028
3×10^{-7}	-	-	-	-	-	-
	-	-	-	-	-	-
		1.25 mM K^+			2.5 mM K^+	
1×10^{-8}	.193	12.3	.0157	.124	12.1	.0102
	.179	12.6	.0142	.110	11.8	.0093
3×10^{-8}	.359	37.7	.0095	.469	38.5	.0122
	.290	37.7	.0077	-	38.3	-
1×10^{-7}	.220	109.	.0020	1.06	107.	.0099
	-	113.	-	.456	114.	.0040
3×10^{-7}	-	351.	-	-	337.	-
	-	339.	-	-	344.	-
		5.0 mM K^+			20 mM K^+	
1×10^{-8}	.248	11.7	.0212	.014	12.4	.0011
	.097	12.9	.0075	.925	12.0	.0771
3×10^{-8}	.483	37.5	.0129	.110	38.8	.0028
	.235	37.4	.0063	-	39.3	-
1×10^{-7}	-	111.	-	1.10	107.	.0103
	.727	108.	.0067	.879	107.	.0082
3×10^{-7}	.355	342.	.0010	-	349.	-
	.963	346.	.0028	2.06	354.	.0058
		Without K^+				
1×10^{-8}	.455	12.2	.0373			
	.331	12.4	.0267			
3×10^{-8}	.649	38.1	.0170			
	.566	37.9	.0149			
1×10^{-7}	.068	107.	.0006			
	1.40	110.	.0127			
3×10^{-7}	-	-	-			
	-	-	-			

TABLE 50. Experimental binding data for rat heart Na^+K^+-ATPase in the absence of K^+

Glycoside Molarity	bound	unbound	b/u	bound	unbound	b/u
Study 1		CYMAROL			OUABAIN	
1×10^{-8}	.173	9.80	.0176	.257	13.7	.0188
	.098	10.3	.0095	.314	14.6	.0215
3×10^{-8}	.420	30.5	.0138	.956	43.6	.0219
	.352	32.1	.0110	.873	43.2	.0202
1×10^{-7}	.781	105.	.0074			
	.355	109.	.0032			
3×10^{-7}	.014	328.	.0000			
	.639	340.	.0019			
Study 2		CONVALLATOXOL			OUABAIN	
1×10^{-8}	2.05	12.9	.159	1.20	13.6	.088
	2.04	13.4	.152	1.11	12.7	.087
3×10^{-8}	2.90	42.1	.069	2.63	40.7	.065
	3.00	41.9	.072	2.64	39.6	.067
1×10^{-7}	4.04	138.	.029	3.97	126.	.032
	3.60	138.	.026	4.24	131.	.032
3×10^{-7}	3.63	442.	.008	4.61	393.	.012
	4.46	436.	.010	4.58	382.	.012

TABLE 51. Experimental binding data for rat heart Na^+K^+-ATPase in the absence of K^+

Glycoside Molarity	bound	unbound	b/u	bound	unbound	b/u
	CONVALLATOXOL			CYMAROL		
1×10^{-8}	.304	10.4	.029	.11	8.78	.012
	.290	10.3	.028	.07	9.18	.008
3×10^{-8}	.8	34.2	.023	.27	28.1	.010
	.704	33.7	.021	.25	29.3	.008
1×10^{-7}	1.57	97.7	.016	0	94.5	-
	1.37	97.4	.014	.78	96.1	.008
3×10^{-7}	.22	317.	.001	1.59	302.	.005
	.52	328.	.002	4.57	304.	.015
	DIGOXIN			DIGITOXIN		
1×10^{-8}	.08	10.2	.008	.20	6.41	.031
	.12	10.1	.012	-	-	-
3×10^{-8}	.29	32.5	.009	.97	21.3	.046
	.53	33.5	.016	1.02	21.3	.048
1×10^{-7}	-	93.7	-	4.01	64.5	.062
	2.69	97.5	.028	7.52	63.8	.118
3×10^{-7}	.09	300.	.0003	32.6	173.	.188
	.58	303.	.002	20.6	208.	.099
	OUABAIN					
1×10^{-8}	.12	12.5	.010			
	.03	12.9	.002			
3×10^{-8}	.50	42.0	.012			
	.45	39.7	.011			
1×10^{-7}	.82	113.	.007			
	.92	115.	.008			
3×10^{-7}	0	350.	-			
	0	348.	-			

TABLE 52. Experimental binding data for rat heart Na^+K^+-ATPase in the presence of 0.625 mM K^+

Glycoside Molarity	bound	unbound	b/u	bound	unbound	b/u
		CONVALLATOXOL			CYMAROL	
1×10^{-8}	.25	10.7	.023	0	8.72	-
	.11	10.3	.011	0	9.22	-
3×10^{-8}	.37	32.8	.011	.04	29.8	.001
	.36	33.1	.011	.07	30.2	.002
1×10^{-7}	.49	96.5	.011	0	91.4	-
	1.01	96.5	.005	.23	97.1	.002
3×10^{-7}	0	313.	-	0	321.	-
	.49	332.	.001	0	317.	-
		DIGOXIN			DIGITOXIN	
1×10^{-8}	.04	10.1	.004	.18	5.63	.032
	.02	10.3	.002	1.20	4.45	.270
3×10^{-8}	1.06	32.1	.033	1.52	20.1	.076
	.16	32.1	.005	.96	20.8	.046
1×10^{-7}	.66	93.4	.007	2.36	49.1	.048
	1.12	97.4	.011	4.58	58.9	.078
3×10^{-7}	5.60	295.	.019	31.8	170.	1.87
	4.39	294.	.015	17.8	198.	.090
		OUABAIN				
1×10^{-8}	.05	13.1	.004			
	.03	12.9	.002			
3×10^{-8}	0	42.1	-			
	.32	40.5	.008			
1×10^{-7}	.47	111.	.004			
	.71	109.	.006			
3×10^{-7}	0	353.	-			
	0	343.	-			